Isolation of Disproportionating Enzyme (EC 2.4.1.25) from Potato and Investigation of its Role in Starch Metabolism

Takeshi Takaha

PhD

University of Edinburgh

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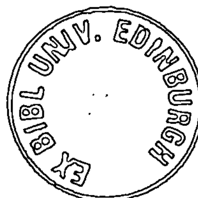

Declaration

I declare that this thesis was composed by myself, and that the work contained within is my own, unless otherwise stated.

Abstract

D-enzyme, disproportionating enzyme, or 4-α-glucanotransferase (EC 2.4.1.25) catalyses glucan transfer from one α-1,4-glucan molecule to another, or to glucose. From the wide distribution of D-enzyme in starch accumulating organs of plants, it is believed that D-enzyme is involved in starch metabolism, but the function *in vivo* is not known. In the present work, D-enzyme was purified from potato tubers, and cDNA and genomic DNA clones for potato D-enzyme were isolated.

The biochemical analysis of purified recombinant D-enzyme suggested that high molecular weight starch (amylose and amylopectin) can serve as donor and acceptor, and very long α–1,4-glucans or even highly branched glucans can be transferred by the enzyme. It was also discovered that D-enzyme catalyses an intra-molecular transglycosylation (cyclisation) reaction on amylose and amylopectin, as well as the well studied inter-molecular transglycosylation (disproportionation) reaction.

Analysis of D-enzyme gene expression was carried out by Northern and Western blot analysis, and in transgenic potato plants using the GUS-reporter gene fusion system. These results suggested that D-enzyme mRNA accumulates under circumstances when starch biosynthesis is most active but declines in amount under conditions when starch is broken down. These observations appear to contradict the widely held view that D-enzyme is involved in starch breakdown, and may suggest a function in starch synthesis. Transgenic potato plants with dramatically reduced D-enzyme activity were obtained by introducing sense and antisense D-enzyme cDNA sequences with the CaMV35S promoter, and various phenotypic changes were observed. These plants grew slower than wild type, produced less leaves, less mass of tubers and the apical meristems suffered necrosis. Furthermore, tubers from these plants sprouted later and the growth of sprouts was slower than wild type. However no significant difference was found in starch produced in tubers, either in its quantity or quality. From all these results and the available information about starch metabolism, possible roles of D-enzyme in starch metabolism are discussed.

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I dedicate this work to my family whose help and support over the years has made it all possible.

Abbreviations

3-PGA	3-phospho glycerate
(v/v)	volume:volme ratio
(w/v)	weight:volume ratio
°C	degree Celsius
A	absorbance
ADP	adenosine 5'-diphosphate
ADP-glc	ADP-glucose
AGPase	ADP-glc pyrophosphorylase
ATP	adenosine 5'-triphosphate
BE	branching enzyme
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
CaMV	Cauliflower mosaic virus
cDNA	complementary DNA
CGTase	cyclodextrin glucanotransferase
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
CTAB	cetyl triethylammonium bromide
C-terminal	carboxy-terminal
Da	dalton
dCTP	2' deoxycytidine 5' triphosphate
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DP	degree of polymerisation
EDTA	ethylenediaminetetraacetic acid (disodium salt)
g	relative centrifugal force
G-1-P	glucose-1-phosphate
GA_3	gibberellic acid
GBSS	granule-bound starch synthase
GST	gluthatione S-transferase
GUS	β-glucuronidase
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IPTG	isopropyl β-D-thiogalactoside
MES	2-(N-morpholino)ethanesulfonic acid
Mr	relative molecular mass
mRNA	messenger RNA
M&S	Murashige and Skoog
MU	methyl umbelliferone
MUG	methyl umbelliferyl glucuronide
NAA	α-naphthaleneacetic acid
NPT	neomycin phosphotransferase
N-terminal	amino-terminal
PAD	pulsed amperometric detector
PAGE	polyacrylamide gel electrophoresis

Pi	inorganic phosphate
polyA	polyadenylation
PVP	polyvinylpyrrolidone
RI	refractive index
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SS	starch synthase
SSS	soluble starch synthase
TBS	tris buffered saline
TEMED	N, N, N', N',-tetramethylethylenediamine
Ti	tumour inducing
TLC	thin-layer chromatography
TNS	triisopropyl-naphthalenesulfonic acid sodium salt
TOF-MS	time of flight mass spectorometer
Tris	tris-(hydroxymethyl)-methylamine
vir	virulence genes on *Agrobacterium tumefaciens* Ti plasmid

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Chapter 1

Introduction

1.1 Starch and its structure

Starch is the dominant storage polysaccharides in plants and is a major metabolic substrate. It is present in most higher plants and in practically every type of tissue; leaves, fruits, pollen grains, roots, shoots and stems. In certain tissues of plants it can accumulate to high levels, for example in cereal grains (for example rice, maize, wheat, sorghum), roots and tubers (cassava, potato) and legume seeds (peas, lentils).

Starch occurs as water-insoluble granules in membrane-bound organelles, the plastids (chloroplasts and amyloplasts). The granules contain principally two different polysaccharides, amylose and amylopectin. Amylose is a linear chain of D-glucose units linked together by α-1,4-linkages. The length of amylose chains among different plant species is variable but usually ranges between 10^2 to 10^4 glucose units (Shannon and Garwood, 1984). A few α-1,6-linked branches may be present (Hizukuri *et al.*, 1981). Amylopectin is a highly branched polymer of glucose, and believed to take the clustered structure (Hizukuri, 1986, 1996, see Figure 1.1). Each cluster unit consists of α-1,4-linked chains (the A, and B1 chains) with degree of polymerisation (DP) of 15-20 joined together by α-1,6-linkages. Cluster units are further linked together by relatively long α-1,4-linked chains (the B2, B3 and B4 chains) with DP ranging from 30 to about 100, to construct the amylopectin molecule which may consist of a total of 10^4 to 10^5 glucose units (Shannon and Garwood, 1984; Hizukuri, 1996). The overall sizes and degree, length and frequency of branching of these molecules, and the ratio of amylose to amylopectin, are subject to genetic and developmental variation.

Starch may be classified into two groups, transitory starch and reserve starch. The former is usually stored inside the chloroplasts of the photosynthetic tissues during the light period and is degraded during the following dark period. Reserve starch is deposited inside the amyloplasts of storage organs, such as seeds, fruits and tubers, over a longer period during one season and remobilised at another.

1.2 Biochemistry, molecular biology and mutation analysis of plant starch synthesis

1.2.1 Introduction

The pathway of starch synthesis is relatively well understood, with three important enzymes having been characterised in detail (Preiss, 1991). ADP glucose (ADP-glc)

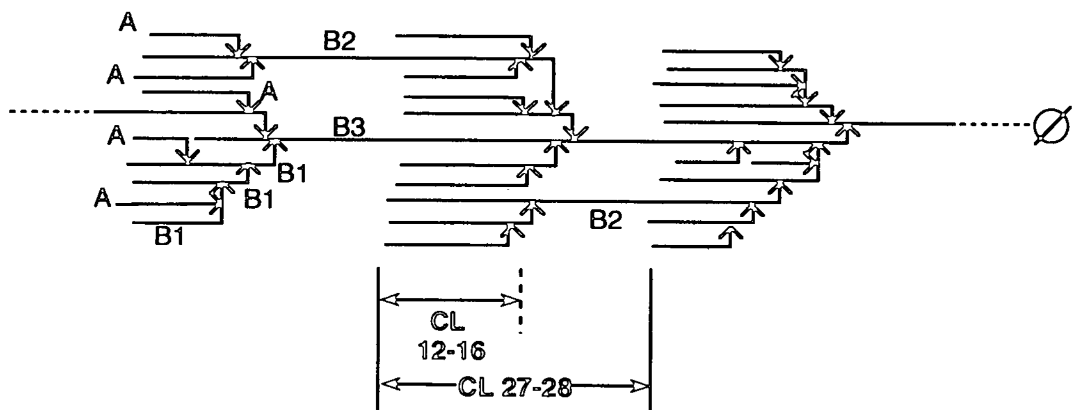


Figure 1.1 Structure of amylopectin.
Diagrammatic representation of the structure of amylopectin (Hizukuri *et al.* 1981). Linear lines indicate α-1,4-glucan chains. Arrows indicate α-1,6-branch points. Ø Glucosyl residue at reducing end. CL, chain length. For details see text (section 1.1).

pyrophosphorylase (EC 2.7.7.27, AGPase) catalyses ADP-glc formation from glucose-1-phosphate and ATP. Starch synthase (EC 2.4.1.21, SS) catalyses glucosyl transfer from ADP-glc to the growing α-1,4-D-glucan chain and starch branching enzyme (EC 2.4.1.18, BE) catalyses a glucanosyl transfer reaction creating α-1,6-linked branches. Starch synthesis is controlled quantitatively in part by the regulation of enzyme synthesis, but is also thought to be regulated to a significant extent by substrates and effectors of AGPase (Preiss, 1991). However, such regulation may differ between plants or between organs in one plant, as described in more detail in the following section. Two major factors to determine the property of starch are the ratio of amylose and amylopectin and the branching structure of amylopectin. Work focused on these issues is also summarised in the following section, although we are still far from a complete understanding.

1.2.2 Enzymes involved in starch synthesis

1.2.2.1 ADP-glucose pyrophosphorylase (AGPase)

AGPase is present in leaves and non-green reserve tissue of higher plants and catalyses the synthesis of the ADP-glc, the substrate for SSs. The plant AGPase has heterotetrameric structure and is composed of two subunit types (large and small), each of which is encoded by a different gene. The enzyme was exclusively located in isolated spinach chloroplasts (Mares *et al.*, 1978). Localisation of the enzyme in amyloplasts in potato tuber was demonstrated by immunocytochemical analysis (Kim *et al.*, 1989). The activity of the enzyme is activated by 3-PGA and inhibited by Pi, and starch synthesis is thought to be controlled to a significant extent by this allosteric regulation of the enzyme (for review, see Preiss, 1991). The importance of AGPase in starch biosynthesis was demonstrated through the analysis of mutants containing lower activity of the enzyme. Maize mutants (*brittle-2* and *shrunken-2*), which contain only 25 - 30 % of the wild-type amount of starch in their endosperm, show only 5 - 10 % of residual activity of AGPase (Tsai and Nelson, 1966; Dickinson and Preiss, 1969). The *adg1* and *adg2* mutants of *Arabidopsis thaliana* which have considerably reduced activity in the leaves (2 % and 5 % respectively), contain only 2 and 40 % starch in their leaves (Lin *et al.*, 1988a; Lin *et al.*, 1988b). A similar mutant (*rb*) was also isolated from pea, and reduced ADP-glucose pyrophosphorylase activity (3-5 %) resulted in reduced starch formation (38 - 72 %) (Smith *et al.*, 1989).

A cDNA clone for each subunit of AGPase has been isolated from many higher plants including wheat leaf and endosperm (Olive *et al.*, 1989), rice seeds (Anderson *et al.*, 1989), maize endosperm (Bae *et al.*, 1990; Bhave *et al.*, 1990), *Arabidopsis* leaf (Villand *et al.*, 1993), barley (Villand *et al.*, 1992a; Villand *et al.*, 1992b) and potato tubers (Müller-Röber *et al.*, 1990; Müller-Röber and Koßmann, 1994). Northern analysis of gene expression suggested that each gene for each subunit is regulated differently in terms of organ specificity (Olive *et al.*, 1989; Giroux and Hannah, 1994; Prioul *et al.*, 1994; Weber *et al.*, 1995),or in response to added sucrose (Müller-Röber *et al.*, 1990). The significance of organ specific expression of isoforms of both subunits of AGPase is not understood, but is potentially very important to produce AGPases with different properties in different organs (Martin and Smith, 1995).

1.2.2.2 Starch synthase (SS)

SS activity is found both in the stroma (soluble starch synthase, SSS) and tightly bound onto the starch granule (granule-bound starch synthase, GBSS). Biochemical and molecular biological analysis indicated that both GBSS and SSS exist as multiple forms. Identification of isoforms from biochemical approaches have been difficult, because breakdown products of the original enzyme seemed to be produced during purification, and considered as isoforms (Marshall *et al.*, 1996). The starch synthase sequences (including both GBSS and SSS) so far available have been recently grouped into three major families (Marshall *et al.*, 1996).

The first family, refered to as the GBSSI family, includes maize GBSSI (Klösgen *et al.*, 1986), rice GBSSI (Okagaki, 1992), wheat GBSSI (Clark *et al.*, 1991), barley GBSSI (Rohde *et al.*, 1988), cassava GBSSI (Salehuzzaman *et al.*, 1993), potato GBSSI (Visser *et al.*, 1989) and pea GBSSI (Dry *et al.*, 1992), and is sometimes referred to as waxy protein. GBSSI family enzymes are nuclear-encoded enzymes of about 60 kDa which are active in starch synthesising plastids (Shannon and Garwood, 1984; Vos-Scheperkeuter *et al.*, 1986). The GBSSI protein catalyses the elongation of both amylose and amylopectin *in vitro* (Leloir *et al.*, 1961). Strong evidence about the role of this enzyme in starch synthesis *in vivo* was obtained from the analysis of mutants. In the *waxy* mutations of maize, there is a drastic reduction in the amylose content of the starch granules (29 % to 0 %) and a concomitant loss of activity of GBSS. The starch grains in the *waxy* mutant do not contain a 60-kDa

polypeptide which is present in wild type grains (Echt and Schwartz, 1981). The overall amount of starch synthesised in the *waxy* mutant is the same as in wild type, suggesting that the GBSSI protein is not required for amylopectin synthesis (Shannon and Garwood, 1984). These data demonstrated that GBSSI is responsible for the synthesis of amylose. Similar mutants were also found in rice (Sano, 1984), barley (Rohde *et al.*, 1988), potato (Hovenkamp-Hermelink *et al.*, 1987), pea (Denyer *et al.*, 1995) and sorghum (Hseih, 1988). An amylose free phenotype was also found in transgenic potato in which GBSSI activity was reduced by introducing an antisense copy of the gene (Visser *et al.*, 1991; Kuipers *et al.*, 1994). The expression of GBSSI genes was analysed at the RNA level in potato (Visser *et al.*, 1989; Dry *et al.*, 1992) and in pea (Dry *et al.*, 1992). The GBSSI mRNA was most abundant in tubers in potato and in developing embryos in pea, but found very weakly in some other organs including leaves. An activation of gene transcription by sucrose is implied from studies with the GUS reporter gene (Visser *et al.*, 1991).

The second family, refered to as the SSII family, includes potato SSII (Edwards *et al.*, 1995), rice SSS (Baba *et al.*, 1993), and pea SSII (previously referred to as GBSSII in Dry *et al.*, (1992)). Enzymes belonging to this family have been well studied in potato tubers and in pea embryos, and shown to have a molecular mass of 77 kDa. In pea embryo, SSII is the major SSS and accounts for 60 to 70 % of the total SSS activity (Denyer *et al.*, 1993). However, in potato tuber, SSII accounts for only 10 to 15 % of the total SSS activity (Edwards *et al.*, 1995). Northern blot analysis showed that SSII mRNA was most abundant in developing pea embryos and in potato tubers (Dry *et al.*, 1992). However, GBSSI and SSII genes were expressed in different stages of embryo development in pea and tuber development in potato (Dry *et al.*, 1992). Mutants for SSII family enzymes have not been isolated from any higher plants, so the role of SSII family enzymes in starch metabolism is still questionable. However, SSII family enzymes are thought to be predominantly involved in the synthesis of the α-1,4-glucan linkages present in amylopectin, since amylose seems to be produced only by GBSSI.

The potato SSIII which has been recently identified as the major SS activity in the soluble fraction of potato tubers (Marshall *et al.*, 1996) is the only member of the third family, refered to as the SSIII family. The SSIII protein is 140 kDa and is distinctly different from any other SS sequences. Transgenic potato plants with reduced SSIII were produced by introducing an antisense copy of the SSIII gene (Marshall *et al.*, 1996). Severe reduction in activity did not affect much the starch content or the ratio of amylose to amylopectin in tubers. However, a profound change

in the morphology of starch granules was produced, indicating the importance of SSIII in determining the structure of starch in the granule (Marshall *et al.*, 1996).

Comparison of catalytic properties of SSS isoforms was carried out by using biochemically identified SSS isoforms from maize (Sivak and Preiss, 1995). These results suggested that one isoform of 70 kDa has a higher preference for the shorter exterior A-chains, and other isoforms of 95 kDa have higher preference for longer chains (Sivak and Preiss, 1995). At the moment, only the GBSSI gene have been isolated from maize, so it is not possible to apply this information obtained from biochemical analysis to the above mentioned SS families which are based on sequence similarity. However, the difference found between SSS isoforms in their catalytic properties is potentially very important for constructing the amylopectin molecule or starch granule.

1.2.2.3 Starch branching enzyme (BE)

The α-1,6 linked branch present in amylopectin is produced by the action of starch branching enzyme (BE). The activity of BE was found exclusively in the chloroplast fraction in spinach leaves (Okita *et al.*, 1979). Multiple forms of BE biochemically identified in a variety of plant tissues (for review, see Preiss, 1988) have been confirmed at the molecular level, since increasing numbers of cDNAs for BE have been isolated. Recently, all the available BE sequences were divided into two families, based on the similarity in their primary sequences (Burton *et al.*, 1995).

The BE family A includes pea SBEI (Burton *et al.*, 1995), maize SBEII (Fisher *et al.*, 1993), rice BE III (Nakamura and Yamanouchi, 1992), potato SBEII (Larsson *et al.*, 1996), *Arabidopsis* SBE2.1 and SBE2.2 (Fisher *et al.*, 1996), and the BE family B includes pea SBEII (Burton *et al.*, 1995), maize SBEI (Baba *et al.*, 1991), rice BEI (Mizuno *et al.*, 1992), cassava SBE (Salehuzzaman *et al.*, 1992) and potato SBEI (referred to as SBE in Poulsen and Kreiberg (1993)). Mutants for BEs belonging to family A have been isolated and well studied in pea and maize. The mutant, wrinkled-seed pea, lacking SBEII, has significantly reduced starch levels (66-75 %) and higher proportion of amylose (60-70 % instead of 33 %), in contrast to embryos of wild type (Edwards *et al.*, 1988; Smith, 1988; Bhattacharyya *et al.*, 1990). The maize SBEII mutant, *amylose-extender*, accumulates starch with increased amylose content (60 %) while the amylose content in wild type maize starch is about 27 to 29 % (Hedman and Boyer, 1982; Stinard *et al.*, 1993). A similar amylose-extender mutant has also been isolated in rice (Mizuno *et al.*, 1993). These

results strongly indicate the important role of BE belonging to family A in amylopectin synthesis. On the other hand, no mutants for BE belonging to family B have been isolated from any higher plants. Introduction of an antisense SBEI (family B) gene into wild type (Müller-Röber and Koßmann, 1994) and into *amf* mutant (Flipse *et al.*, 1996) potato was carried out and the expression of SBEI was successfully reduced to undetectable levels in tubers. However, no significant difference was found in the quantity and quality of starch.

The two BE isoforms may have different catalytic properties as indicated by maize BE isoforms. Purified maize SBEII (family A) has a lower affinity for amylose than purified SBEI (family B) enzyme and transfers shorter chains than those transferred by SBEI (Takeda *et al.*, 1993). Expression analysis of BE genes also suggested that pea SBEI (family A) and SBEII are differently expressed during embryo development (Burton *et al.*, 1995). The differences found between the two BE isoforms in their catalytic properties and timing of their gene expression, is potentially very important for constructing the amylopectin molecule or starch granule.

1.2.3 Quantitative control of starch synthesis

Starch synthesis in photosynthetic tissue appears to be regulated at the level of ADP-glc synthesis. This view was supported by the analysis of an *Arabidopsis* mutant with dramatically reduced levels of AGPase. It was demonstrated that AGPase exercises the most significant degree of control on the pathway from hexose phosphate to starch in the chloroplast (Neuhaus and Stitt, 1990). AGPase has been studied from many plant tissues and has been reported to be activated by 3-PGA, and to be inhibited by inorganic phosphate (for review, see Preiss, 1991). Since a high rate of photosynthesis results in the production of 3-PGA and the utilisation of Pi via photophosphorylation, the stromal 3-PGA : Pi ratio is thought to reflect the availability of fixed carbon for starch synthesis. Thus the regulation by these metabolites allows the rate of starch production to match the supply of substrate provided by photosynthesis.

The regulation of starch synthesis in non photosynthetic starch accumulating organs, may also be regulated at the level of ADP-glc synthesis, through the allosteric regulation of AGPase activity as found in photosynthetic tissue (for review, see Preiss, 1991, 1993). All the mutants with reduced AGPase activity in maize (Tsai and Nelson, 1966) and pea (Smith *et al.*, 1989) have correspondingly reduced starch content in their starch storage organ. In potato, transgenic plants expressing antisense

RNAs for one subunit of AGPase, showed proportional decrease in AGPase activity and starch content (Müller-Röber *et al.*, 1992). Additionally, potato plants transformed with the AGPase gene from mutant *E. coli,* which is insensitive to allosteric regulation, produced tubers with starch increased by 30 to 60 % compared to wild type tubers (Stark *et al.*, 1992). All these results indicate the importance of AGPase and the allosteric regulation of this enzyme in starch synthesis in storage organ, but it is not clear whether AGPase has the greatest control over the rate of starch synthesis in storage organs (Müller-Röber and Koßmann, 1994; Martin and Smith, 1995; Smith *et al.*, 1995). Furthermore, the significance of allosteric regulation of AGPase seems unlikely, because in storage organs, the stromal 3-PGA / Pi ratio is not thought to reflect the availability of fixed carbon for starch synthesis. Although the AGPases purified from some storage organs were reported to be under the control of allosteric regulation by 3-PGA / Pi ratio (Preiss, 1993), it is questionable whether this is a common feature for all AGPases in plants. Molecular analysis of AGPase genes showed that more than one large subunit isoform exists in wheat, barley and potato plants, and expression of their genes is regulated differently in terms of organ specificity (Olive *et al.*, 1989; Villand *et al.*, 1992a; Villand *et al.*, 1992b; Müller-Röber and Koßmann, 1994;),or in response to added sucrose (Müller-Röber *et al.*, 1990). Similar multiple isoforms of small subunits are also reported in bean and in maize, and in both cases, gene expression is regulated differently in terms of organ specificity (Giroux and Hannah, 1994; Prioul *et al.*, 1994; Weber *et al.*, 1995). These results suggest that there are potentially many AGPases with different combinations of isoforms of large and small subunits, and they may have different sensitivity to allosteric regulation (Martin and Smith, 1995).

1.2.4 The control of starch structure

1.2.4.1 Synthesis of amylose

Amylose is the minor (typically 15 to 30 % in weight) component of the starch granule. The synthesis of amylose is relatively well understood from the analysis of amylose free mutants. Such mutants have been obtained in maize (Nelson and Rines, 1962), rice (Sano, 1984), barley (Rohde *et al.*, 1988), potato (Hovenkamp-Hermelink *et al.*, 1987), pea (Denyer *et al.*, 1995) and sorghum (Hseih, 1988). In all these cases, the defective gene product was shown to be the GBSSI protein. These results indicated that GBSSI is responsible for the synthesis of amylose. Interestingly, the overall amount of starch synthesised in the GBSSI mutant of maize is the same as in wild type, suggesting that GBSSI is not required for amylopectin

synthesis (Shannon and Garwood, 1984). The reason why amylose produced by GBSSI is not attacked by BE, and thus remains unbranched, may be explained by the granule-bound nature of the enzyme. GBSSI may be active only inside the granule where soluble BE cannot attack the amylose produced (Denyer *et al.*, 1993).

1.2.4.2 Synthesis of amylopectin

Amylopectin in the major component of starch and is generally believed to adopt the cluster structure (Figure 1.1, for review, see Hizukuri, 1996). The A and B1 chains of amylopectin which comprise the cluster units, may form double helices and add crystallinity to the amylopectin molecule, which may be very important to build the insoluble starch granule. Amylopectin can be distinguished from glycogen, a highly soluble branched glucan found in animals and microorganisms, by the presence of this highly ordered and dense packing of glucan chain. Since bacterial glycogen is synthesised by similar enzyme systems, AGPase, SS (glycogen synthase) and BE (for review, see Preiss and Romeo, 1989), the mechanism in plants to construct this highly ordered amylopectin structure is of great interest.

It has been believed that the structure of amylopectin can be explained by the properties of BEs and SSs. As described before, higher plants generally contain at least two BE isoforms (A, and B families) and at least two or three SS isoforms (GBSSI, SSII and SSIII families). Since GBSSI is responsible for amylose synthesis, other soluble SSs (SSII and SSIII) should be involved in amylopectin synthesis. Progress has been made in maize where two BE isoforms and two SSS isoforms were shown to have different catalytic properties. SBEII (family A) has a lower affinity for amylose than SBEI (family B) and transfers shorter chains than those transferred by SBEI (Takeda *et al.*, 1993). One SSS isoform (70 kDa) has a higher preference for the shorter exterior A-chains, and another SSS isoform (95 kDa) has higher preference for longer chains (Sivak and Preiss, 1995). Differences between isoforms were also found in their gene expression patterns. Pea SBEI (family A) and SBEII are differentially expressed during embryo development (Burton *et al.*, 1995). Although such differences found between two BE isoforms and between two SS isoforms are potentially very important, the synthesis of the amylopectin molecule still cannot be explained.

Recently, strong evidence about the important role of debranching enzyme on amylopectin synthesis and starch granule construction was reported in maize. The *sugary-1* mutant of maize accumulate a reduced amount of starch and a substantial

amount of water soluble glucan which was named phytoglycogen (Sumner and Somers, 1944). Pan and Nelson (1984) found that one of three isoforms of debranching enzyme activity is missing in the endosperm of this mutant, and proposed that the structure of amylopectin is determined by a balance between the actions of branching and debranching enzymes. Recently, the *sugary-1* gene was isolated by transposon tagging, and shown to encode a polypeptide which is highly homologous to a bacterial debranching enzyme (James *et al.*, 1995). A similar mutant was also found in rice that also displayed a decrease in debranching enzyme activity and protein (Nakamura *et al.*, 1996a). However, when the cDNA for rice debranching enzyme was isolated and its chromosomal location was tested, the debranching enzyme gene was mapped on chromosome 4 (Nakamura *et al.*, 1996b), which was different to the chromosome where the *sugary-1* gene had already been mapped (Yano *et al.*, 1984). Nakamura *et al.*, (1996b) then concluded from this result that the *sugary-1* gene is a regulatory gene for debranching enzyme. Although further analysis is necessary to establish the role of debranching enzyme activity in amylopectin synthesis, these results strongly indicate the importance of debranching enzyme activity in the formation of amylopectin and the starch granule.

1.3 Biochemistry, molecular biology and mutation analysis of plant starch breakdown

1.3.1 Introduction

There is a range of enzymes in plants that can contribute to starch breakdown, and two pathways for starch breakdown have been suggested. One is a hydrolytic breakdown by the action of several amylases. α-amylase (EC 3.2.1.1) hydrolyses internal α-1,4-linkage of linear or branched glucans. It is generally believed that α-amylase is the only enzyme that can attack insoluble starch granules and release soluble oligosaccharides (Stitt and Steup, 1985). These solubilised linear or branched oligosaccharides may be further hydrolysed to glucose by α-glucosidase (EC 3.2.1.20) or to maltose by β-amylase (EC 3.2.1.2). Another is a phosphorolytic route by the action of starch phosphorylase (EC 2.4.1.1). This enzyme cleaves α-1,4-linkages of linear or branched glucans to release glucose 1-phosphate from the non-reducing end of the chains. Phosphorylase cannot attack oligosaccharides smaller than maltotetraose, so some other system should be necessary for further breakdown. D-enzyme (EC 2.4.1.25) may convert short oligosaccharides into longer chains upon which phosphorylase can act (see section 1.4). α-1,6-linked branches of starch or

branched oligosaccharides are hydrolysed by debranching enzymes (pullulanase: EC 3.2.1.41; isoamylase; EC 3.2.1.68). The properties of these enzymes from plants are reviewed by Steup (1988) and Manners (1985). Although debranching enzymes have been believed to be involved in starch breakdown, recent studies suggested that they may play important roles upon starch granule synthesis (James *et al.*, 1995; Nakamura *et al.*, 1996a).

Although much is known about the actions of these individual enzymes, little is known about the sequence of events involved in starch breakdown under *in vivo* conditions. Starch is present in most higher plant tissues but the physiological role of the starch in each tissue is apparently different. Thus, the pathway and control of starch breakdown may differ depending upon whether reserve or assimilatory starch is involved. The breakdown of reserve starch in cereal seeds involves the controlled synthesis and secretion of α-amylase (EC 3.2.1.1) which hydrolyses starch to glucose (Beck and Ziegler, 1989). This hydrolytic breakdown occurs in the endosperm tissue which lacks an intact cell structure. In contrast, the mobilisation of plastidic starch, such as in leaves or potato tubers, probably involves phosphorolytic breakdown to glucose-1-phosphate by starch phosphorylase (EC 2.4.1.1). Starch breakdown in plastids may be controlled in part by the synthesis of relevant enzymes, but in contrast to starch synthesis, the regulation of enzyme activity by effector molecules has not been reported, except that high concentrations of Pi will promote phosphorolysis through mass action (Steup *et al.*, 1976).

1.3.2 Cereal seed reserve starch degradation

Starch in cereal seeds is accumulated in the endosperm. During seed germination this reserve starch is broken down to provide energy and building blocks for seedling growth. This starch degradation is extracellular and occurs via the hydrolytic route (Boyer, 1985). The mobilisation of starch is initiated by *de novo* synthesis of hydrolytic enzymes including α-amylases. α-amylase is synthesised primarily in the aleurone layer of the grain, then secreted into the endosperm (Akazawa and Hara-Nishimura, 1985). Total α-amylase activity is the result of several isozymes (Jacobsen *et al.*, 1970; Gale *et al.*, 1983), each encoded by a member of a multigene family (Baulcombe *et al.*, 1987; Khursheed and Rogers, 1988). Although at least three isozymes can be detected in germinated rice grains, a total of ten α-amylase genes have been identified by cloning (Huang *et al.*, 1990) and southern blot analysis (Ranjhan *et al.*, 1991). Northern blot analysis (Karrer and Rodriguez, 1992) and *in*

situ hybridisation experiment (Ranjhan *et al.*, 1992) showed that individual members of the rice α-amylase gene family are differentially expressed in the germinating grain.

Other enzymes involved in hydrolytic breakdown of starch in cereal seeds include β-amylase, pullulanase, isoamylase, glucoamylase and α-glucosidase (Beck and Ziegler, 1989). Especially, the level of β-amylase activity is typically very high in germinated barley seeds, and a maltodextrin-degrading function is evident. However, the seeds of mutants of barley exhibiting only very low levels of β-amylase activity germinate well (Kreis *et al.*, 1987). Thus the function of β-amylase in germination may be insignificant.

The mobilisation of starch is thought to be controlled at the level of enzyme synthesis. The synthesis of α-amylase responds positively to gibberellic acid, and negatively to abscisic acid (Mundy *et al.*, 1986). The *cis* acting region for developmental and hormonal regulation of a rice α-amylase gene was identified in transgenic rice plants using the GUS reporter gene (Itoh *et al.*, 1995). Recently, Yu *et al.* (1991) and Chen *et al.*, (1994) showed that the gene expression of a group of α-amylases in cultured rice cells was induced by carbohydrate starvation and repressed by carbohydrate added to the media. Thus α-amylase synthesis may be controlled by hormonal regulation and /or metabolic regulation.

Imunocytochemical studies revealed that α-amylases in cultured rice cells are localised in starch granules within amyloplasts, in cell walls, and in some of the vacuoles. However, the deduced N-terminal amino acid sequences of nine rice α-amylases do not contain a transit peptide sequence which is necessary for plastid targeting, but they all contain a typical signal sequence which is involved in the translocation of protein across the ER membrane (Chen *et al.*, 1994). These results may suggest the existence of an alternative pathway for import of α-amylases into the plastid (Chen *et al.*, 1994).

1.3.3 Transitory leaf starch degradation

Transitory starch is usually stored inside the chloroplast of the photosynthetic tissues during the light period and is degraded during the following dark period. Thus the degradation of leaf starch is essentially reversible and thought to occur within the plastids of living cells. In this context, leaf starch breakdown is different to cereal seed starch breakdown.

To investigate the pathway of starch degradation, the subcellular distribution of enzymes of starch degradation were analysed in leaves of several plants. Spinach leaf chloroplasts contain α-amylase, debranching enzyme, phosphorylase and D-enzyme (Okita *et al.*, 1979; Okita and Preiss, 1980; Preiss *et al.*, 1980). Pea chloroplasts contain two β-amylases, debranching enzyme, phosphorylase and D-enzyme , but no α-amylase (Kakefuda *et al.*, 1986). However, other studies have demonstrated α-amylase activity in the chloroplasts of pea (Ziegler and Beck, 1986; Ziegler, 1988). *Arabidopsis* leaf chloroplasts contain α-amylase, phosphorylase, D-enzyme and possibly β-amylase (Lin *et al.*, 1988c). Sugar beet leaves contain four endo-amylases and a single debranching enzyme in chroloplasts and one endo-amylase and two exo-amylases outside the chroloplast (Li *et al.*, 1992). These results indicate that leaf transitory starch may be mobilised by the combined activities of α-amylase and starch phosphorylase (Stitt, 1984; Stitt and Steup, 1985), but the overall pathway has not been defined.

It is generally believed that the initial degradation of insoluble starch granules is catalysed by an α-amylase (Stitt and Steup, 1985). This view is based on the following facts. First, in spinach, α-amylase has been the only enzyme demonstrated to attack starch granules isolated from chloroplast (Steup *et al.*, 1983). Second, chloroplastic phosphorylase from spinach and pea appears to be unsuited to degrade large branched starch molecules (Preiss *et al.*, 1980; Steup and Schächtele, 1981; Shimomura *et al.*, 1982), and is effective with starch granules only following hydrolytic digestion (Steup *et al.*, 1983). However, no amylase isolated from the chroloplast has been shown to hydrolyse native starch granules from leaves. On the other hand, Kruger and ap Rees (1983) showed that phosphorylases from pea chloroplasts can release labeled glucose 1-phosphate from ^{14}C-labeled starch granules. α-glucosidase is also reported to attack starch granule (Sun *et al.*, 1995). Further work is necessary to understand this initial event of starch degradation.

Subcellular localisation experiments also indicated that many of the well-studied starch degradative enzymes (e.g. α-amylase, β-amylase and phosphorylase) are located primarily outside of the plastid (Okita *et al.*, 1979; Kakefuda *et al.*, 1986; Lin *et al.*, 1988c; Ziegler, 1988). The presence of β-amylase even in the vacuole of *Arabidopsis*, pea and wheat leaves was also reported (Ziegler and Beck, 1986). Surprisingly, it has recently been reported that a major form of *Arabidopsis* β-amylase is a phloem-specific enzyme (Wang *et al.*, 1995). Since leaf starch is synthesised and accumulates only inside plastids, the role of the abundant extra-chloroplastic forms of these enzymes in starch degradation is questionable.

The regulation of leaf starch degradation is also not understood, but there is some physiological evidence that starch degradation in leaves is regulated. In barley leaves, the degradation of accumulated leaf starch was inhibited when leaves contained a high concentration of sucrose, and began when the concentration of sucrose fell to a particular level (Gordon *et al.*, 1980a; Gordon *et al.*, 1980b). This result suggests the presence of regulatory mechanisms, but knowledge of such mechanisms is scarce (Stitt, 1984; Stitt and Steup, 1985).

Recently, mutants of *Arabidopsis* with altered regulation of starch degradation were identified. One of them is highly deficient in starch degradation in leaves (Caspar *et al.*, 1990). Isolation and biochemical characterisation of these mutants may be a useful approach to the study of leaf starch degradation and its regulation.

1.3.4 Potato tuber reserve starch degradation

Potato tubers accumulate large quantities of starch inside the amyloplasts. Starch breakdown occurs not only at the stage of sprouting but also is initiated by several factors. Starch is frequently converted to high concentrations of soluble sugars such as sucrose, glucose and fructose, as a result of stresses experienced during growth and / or storage (Sowokinos, 1990). Starch granules are degraded intracellularly from the entire particle surface, and electron microscopic analysis showed that during these sweetening processes, starch degradation proceeds inside the apparently intact amyloplast (Isherwood, 1976; Lulai *et al.*, 1986). In this context, potato tuber starch breakdown may be similar to that of leaf starch and not to that of cereal seed starch.

The pathway of starch breakdown and its regulation in potato tubers has not been defined, but there are some reports about individual enzymes. Potato tuber contains at least two forms of phosphorylase. The major form (L type or type I) has a higher affinity for short linear oligosaccharides than high molecular weight branched glucans and is localised in the plastids (Fukui *et al.*, 1987). The minor form (H type or type II) has extraordinarily high affinities for high molecular weight branched glucans including glycogen, and is localised in the cytosol (Fukui *et al.*, 1987). cDNAs for both forms have been cloned and the localisation of the plastid isoform was confirmed by the presence of an amino terminal plastid targeting signal (Nakano *et al.*, 1989; Mori *et al.*, 1991). The physiological significance of cytosolic starch phosphorylase is not understood, however, this isoform has been commonly found in other plant organs, including pea cotyledons (Berkel *et al.*, 1991) and spinach leaves (Shimomura *et al.*, 1982). Northern blot analysis showed that mRNA for plastidic

starch phosphorylase accumulates when and where starch synthesis is active (St-Pierre and Brisson, 1995; St-Pierre *et al.*, 1996). However, further expression analysis at the protein level and in transgenic plants expressing the GUS reporter gene, showed that the plastidic starch phosphorylase gene expression is not only regulated at the transcriptional level but also at the post-transcriptional level (St-Pierre and Brisson, 1995; St-Pierre *et al.*, 1996). Recently, a second plastidic phosphorylase cDNA clone which is highly expressed both in tubers and in leaves has been isolated (Sonnewald *et al.*, 1995). Reduction of this second plastidic starch phosphorylase activity had no significant influence on the accumulation of starch in leaves of transgenic potato plants (Sonnewald *et al.*, 1995). The roles of two plastidic starch phosphorylases in potato are also not understood.

There are conflicting results about potato tuber amylase activities. While α-amylase and β-amylase were clearly resolved by polyacrylamide gel electrophoresis (Siepman and Stegemann, 1967), in another case both α and β-amylases were undetectable (Morrell and ap Rees, 1986). Three forms of debranching enzyme were detected by polyacrylamide gel electrophoresis and purification and characterisation of these enzymes have been performed (Ishizaki *et al.*, 1983). D-enzyme was reported to be present in potato tubers (Peat *et al.*, 1956) and its activity was characterised using a partially purified enzyme (Jones and Whelan, 1969). Nothing is known about the subcellular localisation of α-amylase, β-amylase, debranching enzymes or D-enzyme in potato tubers.

1.4 D-enzyme

1.4.1 D-enzyme in plants

1.4.1.1 Introduction

D-enzyme, disproportionating enzyme, or 4-α-glucanotransferase (EC 2.4.1.25) was first found in potato tubers by Peat *et al.* (1956) but has since been found in carrots (Manners and Rowe, 1969), tomatoes (Manners and Rowe, 1969), germinated barley seeds (Yoshio *et al.*, 1986), sweet potatoes (Suganuma *et al.*, 1991), spinach leaves (Okita *et al.*, 1979), pea leaves (Kakefuda *et al.*, 1986) and *Arabidopsis* leaves (Lin and Preiss, 1988). The enzyme has been partially purified from potato tuber (Jones and Whelan, 1969), barley seeds (Yoshio *et al.*, 1986) and *Arabidopsis* leaves (Lin and Preiss, 1988) and its action has been studied. From the wide distribution in

in plants, D-enzyme is believed to be involved in starch metabolism, however, nothing is known about the function of this enzyme *in vivo.*

1.4.1.2 Action of plant D-enzyme

D-enzyme catalyses glucan transfer from one 1,4-α-D-glucan molecule to another, or to glucose. Maltooligosaccharides are effective donors where the smallest donor molecule is maltotriose (Jones and Whelan, 1969). The upper limit for donor molecules is not known but soluble starch (Yoshio *et al.*, 1986), and amylopectin (Jones and Whelan, 1969; Lin and Preiss, 1988) have been reported to serve as donors. Maltooligosaccharides and glucose serve as acceptors (Jones and Whelan, 1969). Neither the upper limit for acceptors nor the optimal range of acceptors have been studied. Maltose does not function as an acceptor (Jones and Whelan, 1969). Maltooligosaccharides larger than maltose are transferred but glucose is never transferred. Maltosyl groups are transferred more rapidly than any larger group, and the rate of transfer of the maltosyl group to glucose is equal from maltotriose, maltopentaose and maltohexaose (Jones and Whelan, 1969). Maltose is never produced during D-enzyme reaction on any substrate, because there are two "forbidden linkages" in maltooligosaccharides larger then maltotetraose, the non reducing end linkage and the bond penultimate to the reducing end (Jones and Whelan, 1969).

1.1.4.3 Subcellular localisation

The subcellular localisation of D-enzyme was studied in several plant leaves. In pea leaves (Kakefuda *et al.*, 1986), D-enzyme activity was found both in chloroplastic and cytosolic fractions, and the total D-enzyme activity in chloroplastic fraction was higher than that of amylase activity (1.1 : 1) or total phosphorylase activity (8.4 : 1). In *Arabidopsis* leaves (Lin and Preiss, 1988), the activity was found exclusively in the chloroplastic fraction, and again the total D-enzyme activity in the chloroplastic fraction was higher than that of amylase activity (1.5 : 1) or total phosphorylase activity (28 : 1) . In spinach leaves (Okita *et al.*, 1979), D-enzyme activity was found both in the chloroplastic and cytosolic fractions, but the total D-enzyme activity in the chloroplastic fraction was lower than that of amylase activity (0.06 : 1) or total phosphorylase activity (0.25 : 1) . However, Stitt (1984) mentioned that if the similar experiment was carried out at the optimal pH for D-enzyme, the activity in spinach leaves was found exclusively in the chloroplastic fraction.

1.4.2 D-enzyme (amylomaltase) in microorganisms

Amylomaltase catalyses a similar enzymatic reaction to D-enzyme, and is widely distributed in microorganisms. The *E. coli* enzyme is a monomer and has a molecular weight of 78360 (calculated from the deduced amino acid sequence). The action of the enzyme has been extensively analysed by Palmer *et al.* (1976). The difference in enzymatic action of amylomaltase and plant D-enzyme is that, only the former can transfer glucose from donor to acceptor. Amylomaltase is a member of a maltooligosaccharide transport and utilisation system, which includes maltodextrin phosphorylase and maltose transport proteins (Schwartz, 1987). The role of amylomaltase is to convert short oligosaccharides into longer chains upon which maltooligosaccharide phosphorylase can act, since maltodextrin phosphorylase has high affinity for short linear oligosaccharides but cannot act on oligosaccharides smaller than maltotetraose. This model of amylomaltase and maltodextrin phosphorylase function was confirmed by mutant analysis, and the fact that both of these enzymes are encoded by genes on the same operon, *malA*. The gene for amylomaltase has been cloned and sequenced from *E.coli* (Pugsley and Dubrevil, 1988) and *Streptococcus pneumoniae* (Lacks *et al.*, 1982).

The similarity between plastidic starch phosphorylase and *E. coli* maltodextrin phosphorylase as well as that between D-enzyme and amylomaltase may suggest the operation of a similar enzyme system in plants.

1.4.3 Role of D-enzyme in starch metabolism

Although D-enzyme activity has been extensively characterised, its role in plants has not yet been defined. Its wide distribution in plants, its localisation in the chloroplast, and its relatively high activity in the chloroplast, may suggest an important role of this enzyme in starch metabolism. It is generally believed that D-enzyme is involved in starch breakdown, to convert short oligosaccharides into longer chains upon which starch phosphorylase can act (Lee and Whelan, 1971; Lin and Preiss, 1988). Both enzymes are found in plastids (shown above). A similar function has been proposed for *E.coli* where D-enzyme (amylomaltase) and maltodextrin phosphorylase are encoded by genes of the *mal A* operon (shown above). However, there is no experimental result to support this hypothesis. On the other hand, some other roles of D-enzyme may be possible. It may change the structure of starch molecules and grain architecture by modifying chain lengths. It may generate from starch and glucose, oligosaccharides which can serve either as primers for new starch

synthesis, or as substrates for starch branching enzyme, starch phosphorylase or amylases. In order to fully understand the function of D-enzyme, it may be important to know whether starch molecules are effective acceptors and / or donors for glucan transfer.

1.5 Aim of the project

When compared to other starch metabolic enzymes, knowledge about D-enzyme is scarce. The wide distribution in plants and its apparent localisation inside the plastid, potentially suggest an important role for this enzyme in starch metabolism. The initial aim of this project, therefore, is to accumulate knowledge about the function and synthesis of D-enzyme from potato plants. Especially, the analysis of *in vitro* action of D-enzyme on high molecular weight starch are of great interest, since most of the work on this enzyme has been carried out on small oligosaccharides, which are not abundant inside the plastid. The analysis of the synthesis of the D-enzyme at the RNA and protein levels, and their comparison with other starch metabolic enzymes may also suggest the function of D-enzyme. In order to investigate the direct effect of D-enzyme activity *in vivo*, transgenic plants with altered levels of D-enzyme activity should be useful. Such transgenic potato plants can be obtained by introducing a sense or antisense copy of D-enzyme cDNA under the control of the appropriate promoter sequence. All these experiments will be carried out to investigate the function of D-enzyme in starch metabolism

Chapter 2

Materials and methods

2.1 Biological materials

2.1.1 Plant Material

Solanum tuberosum L. (cv. May Queen) was obtained either from a local potato merchant in Osaka, or from the Scottish Office Agriculture and Fisheries Department, Edinburgh. Plants were grown in compost either in a glasshouse or in the growth cabinet at 25 °C with 16 h photo period at an irradiance of 100 µmol $m^{-2}s^{-1}$.

2.1.2 Bacterial strains and genotypes

Escherichia coli:	XL1-Blue	*supE*44 *hsdR*17 *recA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F' [*proAB*$^+$ *lac Iq lacZ*ΔM15 Tn*10*(*tet*^{r})] Used as a host for recombinant manipulation
	TG-1	*supE hsd*Δ5*thi* Δ(*lac-proAB*)/F'[*traD*36 *proAB*$^+$ *lac Iq lacZ*ΔM15] Used as a host for recombinant manipulation
	HB101	*supE*44 *hsdS*20(r_B^- m_B^-) *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1 Used in triparental mating with *Agrobacterium tumefaciens*-carries the helper plasmid pRK2013
	C600*Hfl*	e14$^-$(McrA$^-$) *supE*44 *thi*-1 *thr*-1 *leuB*6 *lacY*1 *tonA*21 *hflA*150::Tn*10* Used as a host for λgt10
	P2-392	e14$^-$(McrA$^-$) *hsdR*514 *supE*44 *supF*58 *lacY*1 or Δ(*lacIZY*)6 *galK*2 *galT*22 *metB*1 *trpR*55 (P2 lysogen) Used as a host for λDash II
	MC1061Δ*glg*	*hsdR mcrB araD139* Δ(*araABC-leu*)7679 Δ*lacX*74 *galU galK rpsL thi* Δ(*asd -glgP*) Kan^r Takata *et al.*, (1995) Used as a host for D-enzyme gene expression

Agrobacterium tumefaciens:

LBA4404	Genotype not available. Carries a cryptic and a 'disarmed' Ti plasmid, the latter lacking the entire T-DNA, but with an intact *vir* region. The bacterial chromosome carries streptomycin resistance and the Ti plasmid carries rifampicin resistance (Hoekema *et al.*, 1983)

2.1.3 Bacterial plasmids and bacteriophage

Vector	Source	Use
pBluescript II SK +/-	Stratagene (La Jolla, CA)	Subcloning
pKK233-2	Pharmacia (Uppsala, Sweden)	Expression in *E. coli*
pGEX-3X	Pharmacia	Expression in *E. coli*
λgt10	Amersham (Buckinghamshire, UK)	cDNA library vector
λDash II	Stratagene	Genomic DNA library vector
pBI101	Clontech (Palo Alto, CA)	Plant transformation
pBI121	Clontech	Plant transformation,
pRK2013	Ditta *et al.*, (1980)	Triparental mating

2.1.4 GBSS cDNA clone

The pUC9 plasmid carrying a 1.1 kb cDNA fragment encoding partial potato granule bound starch synthase cDNA, designated pWx 1.1 (Visser *et al.*, 1991). Gift from R.G.F. Visser, Agricultural University, Wageningen, The Netherlands.

2.1.5 Plastidic starch phosphorylase cDNA clone

The pUC19 plasmid carrying a 0.3 kb *Eco*RI cDNA fragment, which contain 22 bases of the 5'-noncoding region and the coding region corresponding to amino acid -50 to 38 (Nakano *et al.*, 1989). This plasmid is referred to as pUC-PSP in this thesis. Gift from T. Fukui, Osaka University, Japan.

2.2 Miscellaneous

2.2.1 Chemicals

Chemicals and reagents were purchased from BDH Chemicals Ltd. (Poole, UK), Sigma Chemicals Co. Ltd. (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan), unless otherwise stated.

2.2.2 Radiochemicals

α-[^{32}P]dCTP (3000 Ci $mmol^{-1}$) and γ-[^{32}P]ATP (5000 Ci $mmol^{-1}$) were purchased from Amersham International plc (Buckinghamshire, UK).

2.2.3 Autoradiography film

X-ray films were Cronex-4 (Dupont, Frankfurt) and all films were developed in Agfa-Gaevert Gevamatic 60 automatic developer.

2.2.4 Bacteriological media

Luria-Bertani medium ($litre^{-1}$)	10 g bacto-tryptone (Difco laboratories, Detroit, Michigan), 5 g bacto-yeast extract (Difco), 10g NaCl, pH 7.0.
LB agar ($litre^{-1}$)	As for LB medium with the addition of 15 g bacto-agar.
NZY agar ($litre^{-1}$)	5 g bacto-yeast extract , 5 g NaCl, 2 g $MgSO_4$, 10 g casein hydrolysate, 15 g bacto-agar , pH 7.5.
NZY top agar ($litre^{-1}$)	As for NZY agar but with 7 g bacto-agar .

2.2.5 Plant tissue culture media

All media were based on the basic medium of Murashige and Skoog (1962) using M&S medium (Flow Laboratories, Irvine) which does not contain sucrose, IAA and NAA.

Callus induction medium (litre^{-1})	4.7 g M&S medium, 30 g sucrose, 2 mg zeatin riboside, 200 mg NAA, 20 mg GA_3, 8 g bacto-agar, pH 5.7.
Shooting medium (litre^{-1})	4.7 g M&S medium, 30 g sucrose, 2 mg zeatin riboside, 20 mg NAA, 20 mg GA_3, 8 g bacto-agar, pH 5.7.
Rooting medium (litre^{-1})	4.7 g M&S medium, 10 g sucrose, 7 g bacto-agar, pH 5.7.
Shoot culture medium (litre^{-1})	4.7 g M&S medium, 20 g sucrose, 7 g bacto-agar, pH 5.7.

2.2.6 Restriction endonucleases and DNA modification enzymes

All restriction endonucleases and DNA modification enzymes were purchased from Northumbria Biologicals Ltd. (NBL, Northumberland, UK), Boehringer Mannheim (Mannheim, Germany) and Takara Shuzo Co., Ltd.(Shiga, Japan).

2.2.7 Enzymes for carbohydrate analysis

Isoamylase and pullulanase were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Bacterial saccharifying α-amylase was obtained from Nagase Biochemicals (Kyoto, Japan). Glucoamylase from *Rhizopus sp.* was purchased from Toyobo Co., Ltd. (Osaka, Japan).

2.2.8 Carbohydrates

Waxy corn starch was purchased from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). Synthetic amylose with average molecular weight of 5, 10, 30, 70, 110, 320 and 1000 kDa were obtained from Nakano Vinegar Co., Ltd. (Aichi, Japan). Short chain amylose was purchased from Hayashibara Biochemical Laboratories.

2.3 Protein isolation, purification and analysis

2.3.1 Small scale preparation of total protein from plant tissue

About 1 g of frozen tissue of transgenic tubers was ground in a mortar and pestle under liquid nitrogen, and transferred to a centrifuge tube. One ml of extraction buffer

(20 mM Tris-Cl (pH 7.5) containing 5 mM 2-mercaptoethanol) was added to the tube and vortexed, then centrifuged at 4 °C at 11,000 *g* for 5 min. To remove the endogenous sugars in the homogenate, 500 μl of upper aqueous phase was loaded on to a PD-10 column (Pharmacia) and then eluted into 1.2 ml of 100 mM Tris buffer (pH 7.0). This gel-filtered homogenate was used immediately for protein assay, D-enzyme activity assay, activity detection of phosphorylase isozymes after non-SDS PAGE, and immunodetection of D-enzyme protein after SDS-PAGE .

2.3.2 Protein assay

The amount of protein was determined using the Bio-Rad (Hercules, CA) protein determination kit, which is based on the Bradford protocol (Bradford, 1976). BSA was used as the standard protein.

2.3.3 D-enzyme assay

D-enzyme activity was assayed in 100 μl reaction mixture containing 100 mM Tris-Cl buffer (pH 7.0), 5 mM 2-mercaptoethanol, 1 % (w/v) maltotriose and enzyme. The reaction mixture was incubated at 37 °C for 10 min and terminated by immersing the reaction tubes in boiling water for 3 min. Released glucose was measured by the glucose oxidase method (Miwa *et al.*, 1972). One unit of activity is defined as the amount of enzyme which produces 1 μmol of glucose per min under these assay conditions.

2.3.4 SDS-PAGE and measurement of molecular weight

SDS-PAGE was carried out essentially as described in Laemmli (1970). The relative molecular mass of protein was estimated using prestained SDS-PAGE standards (Bio-Rad) as molecular weight markers.

2.3.5 Transfer of protein in the gel to Hybond-ECL membrane

Transfer of proteins in the gel containing SDS to Hybond-ECL (Amersham) was carried out essentially as described by Harlow and Lane (1988). After SDS-PAGE, the gel was incubated in transfer buffer (50 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, 20 % (v/v) methanol) for 15 min. Hybond -ECL membrane, cut to the size of the gel, was immersed in distilled water for 10 min and transferred to transfer buffer for 15 min. The gel, the membrane and Whatman 3 MM paper was assembled into the transfer apparatus. Transfer was carried out at 25 V for 16 h in the transfer buffer

mentioned above. After the transfer, the membrane was rinsed 3 times for 5 min with distilled water and immediately used for immunodetection.

2.3.6 Immunodetection of D-enzyme on the membrane

All steps for immunodetection were carried out at room temperature on a shaking platform. The membrane was incubated in blocking solution (TBS buffer containing 0.1 % (v/v) Tween-20 and 5 % (w/v) dried milk) for 1 h, and washed three times with TBST (TBS buffer containing 0.1 % (v/v) Tween-20). The membrane was then incubated with antibody for D-enzyme diluted in TBST for 1-2 h. The membrane was subsequently washed four times with washing solution for 15 min, then incubated with secondary antibody (anti-rabbit IgG conjugated with horseradish peroxidase) diluted in washing solution for 20 min. Unbound secondary antibody was removed with four 5 min washes in TBST.

Detection of antigens was carried out using ECL western detection reagents (Amersham), and Hyperfilm-ECL (Amersham) according to the manufacturers recommendations.

2.3.7 Native PAGE and phosphorylase activity staining

Separation of isozymes of starch phosphorylase on glucan containing native PAGE and the detection of phosphorylase activities on the gel were carried out as described in Steup (1990). Higher plant phosphorylases have been classified into two types from their intracellular localisation, kinetic properties and affinity for glucans (section 1.3.4). Cytosolic phosphorylase has high affinity towards branched polyglucans (such as glycogen) and moves very slowly in PAGE containing glycogen, whereas the plastidic form has low affinity for such glucans and moves faster in PAGE containing glycogen. Using these different properties of isozymes and the ability of phosphorylases to synthesise amylose in the gel from G-1-P, the activity of each phosphorylase isozyme can be distinctively detected on the gel.

The separation gel was prepared to be 7.5% (w/v) acrylamide, 75 mM borate buffer (pH 7.9), 0.1 % (w/v) ammonium persulphate, 0.1 % (w/v) glycogen, 0.007 % (v/v) TEMED. The extracted protein from each tissue (typically; 20 μg of total protein from potato tuber, and 70-100 μg of total protein from potato leaf tissue) was loaded onto the gel with glycerol and BPB (final concentration 10 % (v/v), and 25 μg ml^{-1}, respectively). The electrophoresis was carried out using the running buffer (75 mM borate buffer, pH 7.9) for about 30-90 min at 100 V at 4 °C. Following

electrophoresis, the gel was first equilibrated with 0.1 M MES buffer (pH 6.0) at 4 °C for 30 min, then transferred to activity staining solution (0.1 M MES (pH 6.0), 20 mM G-1-P, and 0.05 % (w/v) soluble starch) and incubated for 60 min at 37 °C. After the incubation, the gel was carefully washed with distilled water and stained with iodine solution (2 % (w/v) KI, 0.2 % (w/v) I_2). The amylose synthesised on the gel was stained blue or violet in colour.

2.3.8 GUS activity assay

The assay was performed as described in Jefferson *et al.* (1987). About 1 g of frozen tissue was ground in a mortar and pestle under liquid nitrogen, and transferred to a centrifuge tube. One ml of GUS extraction buffer (50 mM sodium phosphate (pH 7.5), 10 mM 2-mercaptoethanol, 0.1 % (w/v) Triton X-100, 1 mM EDTA) was added to the tube and vortexed, then centrifuged at 11,000 *g* for 5 min at 4 °C . The upper aqueous phase was transferred to a new tube and used for protein and GUS activity assays after appropriate dilution with GUS extraction buffer.

4-methylumbelliferyl β-D-glucuronide (MUG), which is cleaved by GUS to yield glucuronic acid and 7-hydroxyl-4-methylcoumarin (4-methylumebelliferone, MU), a fluorescent product, was used as a substrate for GUS assay. To start the enzyme reaction, 200 μl of 5 mM MUG in GUS extraction buffer was added into 800 μl of diluted enzyme solution and incubated at 37 °C. After 0, 5, 10, 15 and 30 min of incubation, 200 μl of reaction mixture was taken into 800 μl of GUS-stop buffer (0.2 M Na_2CO_3). The concentration of MU was determined fluorometrically using a Perkin-Elmer LS series spectrofluorometer with settings of excitation at 365 nm and emission at 455 nm.

2.3.9 Purification of D-enzyme from potato tuber

Potato tubers (2.5 kg) were washed, peeled and homogenised in the presence of 20 mM Tris-Cl (pH 7.5) and 5 mM 2-mercaptoethanol. After centrifugation and filtration through 0.45 μm membrane, the homogenate was applied to a Q-Sepharose fast flow column (16 x 100 mm, Pharmacia) and washed with 150 mM NaCl in 20 mM Tris-Cl (pH 7.5) containing 5 mM 2-mercaptoethanol (buffer A). D-enzyme was eluted with buffer A containing 450 mM NaCl, and dialysed against buffer A. Concentrated Tris buffer, ammonium sulfate and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate and 5 mM 2-mercaptoethanol. The solution was applied to a Phenyl-TOYOPEARL 650M (TOSOH, Tokyo, Japan) column (10 x 100 mm) and eluted with

a linear gradient of 500 to 0 mM ammonium sulfate in buffer A. Active fractions were pooled and dialysed against buffer A. The dialysate was concentrated by Amicon centricon 30 microconcentrator, and applied to a PL-SAX HPLC-column (Polymer Laboratories, UK). The enzyme was eluted with a linear gradient of 150 to 400 mM NaCl in buffer A. Active fractions were pooled and concentrated by centricon 30 microconcentrator.

2.3.10 Amino acid sequencing analysis

Purified D-enzyme was subjected to reverse-phase HPLC using a C4-column (250 x 4.6 mm; YMC Biochemicals, Kyoto, Japan) and eluted with a linear 0-40 % (v/v) gradient of acetonitrile with 0.1 % (v/v) trifluoroacetic acid and freeze dried. 500 pmol of the protein were used for N-terminal amino acid sequencing using an automated peptide sequencer (Shimadzu, Kyoto, Japan). 2 nmol of the protein was digested with TPCK-trypsin (Sigma) in the presence of 2 M urea and 100 mM Tris-Cl (pH 8.0). The fragments generated were separated by reverse-phase HPLC using C4 and C18-columns (250 x 4.6 mm; YMC Biochemicals) with a gradient of acetonitrile containing 0.1 % (v/v) trifluoroacetic acid. Two fragments that were well separated from others were collected and lyophilised, then used for amino acid sequencing using an automated peptide sequencer (Shimadzu, Japan).

2.4 Carbohydrate analysis

2.4.1 General analytical methods

Reducing and non-reducing ends were quantitated using a modified Park-Johnson method (Hizukuri *et al.*, 1981) and the rapid Smith-degradation method (Hizukuri and Osaki, 1978), respectively. Quantitation of glucose was carried out by the glucose oxidase method (Miwa *et al.*, 1972). Total carbohydrate was determined by phenol-sulphuric method (Dubois *et al.*, 1956).

2.4.2 TLC analysis of oligosaccharides

Up to 20 μl of carbohydrate solution (containing 5 to 10 μg of glucan) were spotted on Silica Gel 60 thin layer plates (Merck) and developed three times in 1-butanol/ethanol/water (5:5:3). After development, the TLC plate was dried by incubating at 90 °C, then compounds on the plate were detected by spraying 6 M sulphuric acid in methanol and baking at 140 °C for 3 - 10 min.

2.4.3 Gel-filtration analysis of carbohydrates and estimation of molecular weight

Gel-filtration chromatography of glucan was carried out using a Superose 6 prep grade column (Pharmacia, 10 mm x 300 mm), Superose 12 prep grade column (Pharmacia, 10 mm x 300 mm), Superdex 75 column (Pharmacia, 10 mm x 300 mm) or Superdex 30 column (Pharmacia, 10 mm x 300 mm), or combinations of two of these four columns. The useful fractionation range for each gel-filtration medium is shown below. Two hundred and fifty μl of 0.2 % (w/v) glucan solution was loaded onto the column and eluted with 150 mM sodium acetate at a flow rate of 0.5 to 1.0 ml min^{-1}. The glucan was detected by refractive index detector (Shimadzu). Molecular weight of the glucan was estimated using a standard curve produced with synthetic amylose with average molecular weight of 5, 10, 30, 70, 320 and 1000 kDa as standards.

Medium	Useful fractionation range (Da)	
	Proteins	Dextrins
Superose 6 prep grade	5×10^3 - 5×10^6	up to 1×10^6
Superose 12 prep grade	1×10^3 - 3×10^5	up to 3×10^5
Superdex 75	3×10^3 - 7×10^4	5×10^2 - 3×10^4
Superdex 30	up to 1×10^4	

2.4.4 Absorption spectrum of amylose-iodine complex

One hundred μl of 0.1 % (w/v) glucan solution was mixed with 2 ml of iodine reagent and the absorption spectrum was monitored using a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Iodine reagent was made daily from 0.5 ml of iodine stock solution (0.26 g of I_2 and 2.6 g of KI in 10 ml of water) mixed with 0.5 ml of 1 N HCl and diluted to 130 ml with distilled water.

2.4.5 High performance anion exchange chromatography (HPAEC)

HPAEC was carried out with a DIONEX DX-300 system (Dionex, Sunnyvale, CA) with a pulsed amperometric detector (model PAD-II, Dionex). For analytical purposes, a Carbopac PA-100 column (4 mm x 250 mm) was used. A sample (25 - 250 μl) containing 25 - 100 μg glucan was injected and eluted with a gradient of sodium acetate (0 - 2 min, 50 mM; 2 - 37 min, increasing from 50 mM to 350 mM

with the installed gradient program 3; 37 - 45 min, increasing from 350 mM to 850 mM with the installed gradient program 7; 45 - 47 min, 850 mM) in 150 mM NaOH with a flow rate of 1 ml min^{-1}.

2.4.6 Time of flight mass spectrometry (TOF-MS)

Molecular mass of the glucan mixture was analysed by a Kompact Maldi I TOF-MS system (Shimadzu).

2.4.7 Analysis of the action of D-enzyme on amylose AS-320

2.4.7.1 Preparation of amylose solution and treatment with D-enzyme

Synthetic amylose (20 mg) was dissolved in 2 ml of 1N NaOH solution then neautralised by adding 4 ml of distilled water, 2 ml of 1M sodium citrate buffer (pH 7.0) and 2 ml of 1N HCl. The solution was used immediately after neutralisation for D-enzyme treatment.

To the amylose solution, 68 units of purified D-enzyme from recombinant *E. coli* (section 2.8.2), was added then incubated at 30 °C. The reaction was terminated by boiling for 5 min and the supernatant recovered by centrifugation.

2.4.7.2 Preparation of glucoamylase-resistant molecules

Glucoamylase hydrolyses linear glucan to glucose from its non-reducing end, but cannot attack cyclic glucans. To investigate the presence of cyclic glucan in the reaction mixture from previous section, 100 units of glucoamylase was added and incubated for 4 h at 40 °C. The resultant glucoamylase-resistant glucan was precipitated with 10 volumes of ethanol and lyophilised. This glucoamylase treatment and ethanol precipitation can be repeated, if necessary.

2.4.7.3 Quantitative analysis of glucoamylase-resistant glucan

The quantitative analysis of glucoamylase-resistant molecules in the presence of linear glucan was carried out as follows. The glucan (100 μg) was incubated either with glucoamylase (10 units) only or with glucoamylase (10 units) and α-amylase (10 units) in 100 mM acetate buffer (pH 5.5) for 1 h at 40 °C. After terminating the reaction by boiling for 5 min, released glucose was measured by the glucose oxidase method. The amount of glucoamylase-resistant molecules was calculated by

subtracting the amount of glucose released by glucoamylase and α-amylase from that by glucoamylase only.

2.4.7.4 Size fractionation of glucoamylase-resistant glucan by gel-filtration column

The glucoamylase-resistant glucan was dissolved in 250 μl distilled water then size fractionated by gel filtration chromatography using the Superdex 30 column (Pharmacia, 10 mm x 300 mm) with flow rate of 0.5 ml min^{-1}. The eluate from the column was fractionated, and the glucan in each fraction was analysed quantitatively by the phenol-sulphate method and qualitatively by HPAEC (section 2.4.5).

2.4.7.5 Purification of cyclic α-1,4-glucan with single DP by HPAEC

The size fractionated glucoamylase-resistant molecule (1 mg) was injected onto a Carbopac PA-1 column (9 mm x 250 mm) and eluted with a gradient of sodium acetate (0 - 2 min, 230 mM; 2 - 37 min, increasing from 230 mM to 280 mM with the installed gradient program 6; 37 - 45 min, 280 mM; 45 - 47 min, 850 mM) in 150 mM NaOH, with a flow rate of 5 ml min^{-1}. Eluate from the column was divided for PAD detection and for sample preparation. Eluate for each peak was collected, neutralised with 1 N HCl and precipitated with ten volumes of ethanol and lyophilised.

2.4.7.6 Determination of degree of polymerisation of purified cyclic α-1,4-glucan by partial acid hydrolysis

Purified glucan (~50 μg) for each peak in previous section was resuspended in 20 μl distilled water. A 10 μl aliquot of glucan solution was mixed with 10 μl of 0.2 N HCl and incubated for 15 min at 100 °C. Acid hydrolysis was terminated by adding 240 μl ice cold 150 mM NaOH and immediately analysed by HPAEC with a Carbopac PA-100 column (section 2.4.5).

2.4.8 Analysis of the action of D-enzyme on amylopectin

2.4.8.1 Preparation of amylopectin solution and treatment with D-enzyme

Two grams of waxy corn starch were suspended in 10 ml of distilled water then 90 ml of DMSO was added and mixed well. This viscous amylopectin solution was boiled for 5 min then left overnight at room temperature for complete solubilisation. This stock solution is stable at room temperature. For D-enzyme treatment, 1 ml of

this stock amylopectin solution was incubated in 10 ml of 10 mM sodium citrate buffer (pH 7.0) containing 340 units of purified D-enzyme from recombinant *E. coli* (section 2.8.2), at 30 °C for up to 40 h. During the reaction, 600 μl of reaction mixture was taken and the reaction terminated by boiling for 5 min, then analysed by gel-filtration chromatography using the Superose 6 column plus Superdex 30 column with flow rate of 1.0 ml min^{-1}.

2.4.8.2 Quantitation of non-cyclic, unbranched cyclic and branched cyclic products (see Figure 4.16)

The glucan of interest was dissolved in water to a concentration of 0.2 % (w/v), and 50 μl of the glucan solution was incubated with 450 μl of the following three enzyme solutions containing different combinations of amylases in 100 mM sodium acetate buffer (pH 5.5): (1) glucoamylase (5 units), (2) glucoamylase (5 units), isoamylase (0.2 units) and pullulanase (0.1 units), (3) glucoamylase (5 units) and α-amylase (1 unit), for 4 h at 40 °C. After terminating the reaction by boiling for 5 min, released glucose was measured by the glucose oxidase method. The amount of glucan not involved in cyclic structure, glucan involved in cyclic structure, glucan involved in α-1,4-linked cyclic structure or glucan involved in α-1,4-, and α-1,6-linked cyclic structure was calculated from the following equation, where x, y and z represent the amount of glucose produced by treatment with glucoamylase, glucoamylase and debranching enzymes (isoamylase and pullulanase), and glucoamylase and α-amylase, respectively.

(glucan not involved in cyclic structure) $=100 \times (x / z)$

(glucan involved in cyclic structure) $=100 \times (z - x) / z$

(glucan involved in α-1,4-linked cyclic structure) $=100 \times (z - y) / z$

(glucan involved in α-1,4-, and α-1,6-linked cyclic structure) $=100 \times (y - x) / z$

2.4.9 Analysis of starch in transgenic plants

2.4.9.1 Quantitation of total amount of starch in potato tuber

Tubers were washed, peeled, and sliced then frozen with liquid nitrogen. About 1 g of frozen tissue slices were ground under liquid nitrogen either using a mortar and

pestle or coffee grinder. The frozen tissue powder was transferred to a centrifuge tube containing 5 ml of 80 % (v/v) ethanol put on a balance, and the weight of added tissue was measured. The centrifuge tube was incubated at 80 °C for 30 min with occasional vortexing, then centrifuged at 4,000 *g* for 15 min and the supernatant discarded. This washing of pellet with 80 % (v/v) ethanol was repeated again at 80 °C, and centrifuged. The supernatant was discarded and the pellet was solubilised in 12 ml of DMSO and boiled for 30 min. After cooling, 3 ml of 8 N HCl was added and incubated for a further 30 min at 60 °C, then left overnight at room temperature to obtain complete solubilisation of starch. On the following day, the sample was centrifuged at 4,000 *g* for 15 min, then 3 ml of supernatant was transferred to a new centrifuge tube and the starch was obtained as a pellet by adding 12 ml of ethanol and centrifuging. The starch pellet was solubilised in DMSO then a portion was treated with glucoamylase and α-amylase and the released glucose was measured by the glucose oxidase method.

2.4.9.2 Isolation of starch granule from tubers

Tubers were washed, peeled, and placed in a beaker. Tissue was completely homogenised on ice in cold distilled water with a hand-held blender. The homogenate was filtered through 3 layers of muslin into a second beaker on ice. Starch grains were allowed to settle for 30 min on ice, and then supernatant was poured off. This washing procedure was repeated 4 times. The starch was then resuspended in acetone and transferred to a centrifuge tube. The starch granules were again allowed to settle and acetone was then poured off. This washing with acetone was repeated three times. Finally starch granules were dried at 40 °C in a rotary evaporator.

2.4.9.3 Solubilisation of starch in 90 % (v/v) DMSO

Starch granule (400 mg) was suspended well in 4 ml of cold distilled water and 36 ml of DMSO was then added and immediately vortexed vigorously then left overnight at room temperature to obtain starch dispersion in DMSO. On the following day, the starch solution was boiled for 20 min to obtain the complete solubilisation of starch then left for at least a week at room temperature.

2.4.9.4 Amylose content

Estimation of amylose content was carried out by the method of Hovenkamp-Hermelink *et al.*, (1988). One hundred μl of starch solution in 90 % (v/v) DMSO was mixed with 900 μl of distilled water and vortexed, then mixed with 20 ml of iodine

reagent. The absorbances at 618 and 550 nm were measured using a Shimadzu UV-240 spectrophotometer (Kyoto, Japan). Amylose content was calculated using the following equation.

$$\text{Amylose (\%)} = (3.5 - 5.1 \times R) / (10.4 \times R - 19.9)$$

where $R = A618\ nm / A550\ nm$

Iodine reagent was made daily from 0.5 ml of iodine stock solution (0.26 g of I_2 and 2.6 g of KI in 10 ml of water) mixed with 0.5 ml of 1 N HCl and diluted to 130 ml with distilled water.

2.4.9.5 Analysis of debranched glucans

For debranching of starch, 500 μl of starch solution prepared in section 2.4.9.3 was mixed with 4.5 ml of 20 mM acetate buffer (pH 3.5) and 4.5 μl of isoamylase, then incubated overnight at 40 °C. After incubation the reaction was terminated by boiling for 10 min, then centrifuged to remove denatured isoamylase. The debranched glucan in the supernatant was precipitated with 6 volumes of ethanol, and obtained as a dry pellet by centrifugation and rotary evaporation. The debranched glucan was dissolved in 75 μl of 1 N NaOH then 425 μl of distilled water was added. This debranched glucan solution was analysed by HPAEC or by gel-filtration chromatography using Superdex 75 column (Pharmacia, 10 x 300 mm).

2.4.9.6 Detection of starch in leaves

Leaves were placed in boiling water for two min, soaked in 95 % ethanol until white, washed with water and then incubated in iodine solution (2 % (w/v) KI, 0.2 % (w/v) I_2) for few minutes. Starch in leaves was stained blue in colour.

2.5 DNA isolation, manipulation and analysis

2.5.1 General comments

Unless otherwise stated, all DNA manipulations including small scale plasmid preparation, restriction or modification enzyme treatment of DNA, DNA gel electrophoresis, isolation of DNA fragment from agarose gel, and transformation or transfection of *E. coli* with plasmid or phage DNA, were carried out as described by Sambrook *et al.*, (1989).

2.5.1.1 Large scale plasmid isolation

Preparation of plasmid DNA from 100 to 200 ml of *E.coli* culture was carried out using a Qiagen plasmid midi system (Qiagen Inc., Studio City, CA) according to the manufacturers recommendations.

2.5.1.2 Bacteriophage DNA isolation

Preparation of phage DNA from 10 ml of plate lysate was carried out using Qiagen λ mini system (Qiagen) according to the manufacturers recommendations.

2.5.2 DNA sequencing

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.* (1977) using a T7 Sequencing kit (Pharmacia).

2.5.3 Generation of nested deletions

Nested deletions of DNA fragments subcloned in plasmid were carried out using a Nested deletion kit (Stratagene) according to the manufacturers recommendations.

2.5.4 Radiolabelling of DNA fragment

2.5.4.1 Labelling of ds DNA fragment

Labelling of double-stranded DNA fragment with [α-^{32}P]dCTP was carried out by the random primer method (Feinberg and Vogelstein , 1983). The DNA fragment (30 to 50 ng in 34 μl) was heat denatured at 100 °C for 3 min then quenched on ice. To the denatured DNA, 10 μl of 5X OLB buffer (250 mM Tris-Cl (pH 8.0), 25 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 1 M HEPES (pH 6.6), 1 mg ml^{-1} random hexanucleotides), 2 μl of 10 mg ml^{-1} BSA solution, 3 μl of [α-^{32}P]dCTP (30 uCi), and 1 μl (1 unit) of DNA polymerase I - Klenow fragment were added and incubated for 60 min at 37 °C and the reaction terminated by addition of 200 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Unincorporated nucleotides were removed by passage through a Sephadex G-50 column (5 mm x 30 mm).

2.5.4.2 Labelling of oligonucleotides

Synthetic oligonucleotides were labelled by the kinase reaction of bacterial T4 polynucleotide kinase (PNK). The reaction mixture containing 3 μl of

oligonucleotides (20 ng), 1 μl of 10 x PNK buffer (0.5 M Tris-HCl (pH 7.6), 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA), 5 μl of [γ-^{32}P]ATP (30 uCi), and 1 μl of PNK was prepared and incubated at 37 °C for 60 min. Unincorporated [γ-^{32}P]ATP was removed by passage through a Sephadex G-25 column (5 x 30 mm).

2.6 RNA isolation and analysis

2.6.1 Isolation of total RNA from potato plant tissue

Total RNA was extracted from frozen plant materials by the following methods. About 1 g of frozen tissue was ground in a mortar and pestle under liquid nitrogen, and transferred to a centrifuge tube. 4 ml of extraction buffer (100 mM Tris-Cl (pH 8.5), 10 % (w/v) TNS (Kodak, New Haven, CT) and 6 % (w/v) 4-aminosalicylic acid) and 4 ml of phenol : chloroform (1:1 by volume) were added to the tube and vortexed, then centrifuged at 2,000 *g* for 20 min. The upper aqueous phase was transferred to a new centrifuge tube and precipitated with one-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The precipitate was collected by centrifugation and dissolved in 1 ml of DEPC treated water. RNA was selectively precipitated by addition of an equal volume of 5 M LiCl, and incubation at 4 °C overnight. The RNA precipitated was collected by centrifugation, washed with 70 % (v/v) ethanol and dried.

2.6.2 Horizontal gel electrophoresis of RNA and Northern blotting

RNA was electrophoresed on formaldehyde agarose gels (Maniatis *et al.*, 1982) and blotted onto Hybond N membrane filters (Amersham). The filters were hybridised with a ^{32}P-labelled DNA probes (1 ng ml^{-1}) in 6 x SSPE (900 mM NaCl, 60 mM sodium hydrogen orthophosphate, 6 mM EDTA, pH 7.7) containing 5 x Denhardt's solution, 40 % (v/v) formamide, 0.1 % (w/v) SDS and 0.05 mg ml^{-1} denatured salmon sperm DNA, at 42 °C for 18 h. ^{32}P-labelled DNA probes were prepared as described in section 2.5.4.1. The filters were washed twice in 1 x SSPE containing 0.1 % (w/v) SDS at 42 °C for 15 min and autoradiographed.

The DNA fragment used for probes were,

D-enzyme	1.0 kb *Eco*R I fragment of pDPE-102P
Granule bound starch synthase	1.1 kb *Eco*R I fragment of pWx 1.1 (section 2.1.4)

Plastidic starch phosphorylase	0.3 kb *Eco*R I fragment of pUC-PSP (section 2.1.5)

2.7 Construction and screening of libraries

2.7.1 Construction and screening of potato immature tuber cDNA library

poly(A)$^+$RNA was purified from total RNA prepared from immature potato tubers (1 cm) using the mRNA purification Kit (Pharmacia). cDNA was synthesised from poly(A)$^+$ RNA using the cDNA Synthesis Kit (Pharmacia). This cDNA was ligated into lambda gt10 arms via *Eco*RI /*Not* I adapters and packaged using Gigapack II Gold packaging extracts (Stratagene). The cDNA library was plated on *E.coli* C600*Hfl* cells and transferred to Hybond N$^+$ membrane filters (Amersham). Oligonucleotide hybridisation was done in 6 x SSC (900 mM NaCL, 90 mM sodium citrate) containing 5 x Denhardt's solution, 0.1 % (w/v) SDS, 0.05 mg ml^{-1} denatured salmon sperm DNA and ^{32}P-labelled synthetic oligonucleotide probe (section 2.5.4.2) at 35 °C overnight. The filters were washed twice in 6 x SSC containing 0.1 % (w/v) SDS at 35 °C for 15 min and autoradiographed.

Phage DNA for positive plaques was purified by the plate lysate method (Maniatis *et al.*, 1982) as described in section 2.5.1.2, and the cDNA insert was digested with *Not* I and subcloned into the *Not* I site of pBluescript II SK+ (Stratagene) in both orientations.

2.7.2 Construction and screening of potato genomic DNA libraries

Young potato leaves (5 g) were ground to a fine powder using a mortar and pestle and transferred to a centrifuge tube containing 5 ml of 2 x CTAB (2 % (w/v) CTAB, 0.1 M Tris-Cl (pH 8.0), 1.4 M NaCl, 1 % (w/v) PVP) which has been kept at 70 °C. The tube was vortexed then kept at 55 °C for 10 min, then 5 ml of chloroform:isoamyl alcohol (24:1 by volume) was added and shaken slowly for 30 min at room temperature. The tube was centrifuged and the supernatant transferred to a new centrifuge tube, then mixed with 1/10 volume of 10 % (w/v) CTAB solution. Equal volume of the precipitation buffer (1 % (w/v) CTAB, 0.1 M Tris-Cl (pH 8.0), 10 mM EDTA) was added to the tube and left for 30 min at room temperature, then centrifuged. The supernatant was discarded and the pellet was dissolved in 5 ml of 1

M NaCl in TE buffer at 55 °C, then 5 ml of isopropanol added to precipitate DNA. The DNA pellet was washed with 70 % (v/v) ethanol and dissolved in 3 ml of TE buffer at 55 °C then treated with RNase (at final concentration of 1 μg ml^{-1}).

The purified total DNA was partially digested with *Sau*3A I and DNA fragments about 10-20 kb were purified by sucrose density gradient ultra centrifugation (Maniatis *et al.*, 1982), and ligated into CIAP-treated lambda Dash II (Stratagene) vector arms and packaged using Gigapack II Gold packaging extracts (Stratagene). The genomic DNA library thus obtained was plated on *E.coli* P2-392 cells and transferred to Hybond N+ membrane filters (Amersham). The filters were hybridised with a ^{32}P-labelled full length cDNA probe in buffer containing 6 x SSPE, 5 x Denhardt's solution, 0.1 % (w/v) SDS and 0.05 mg ml^{-1} denatured salmon sperm DNA, at 65 °C for 18 h. ^{32}P-labelled DNA probes were prepared using a random oligonucleotide primer method (section 2.5.4.1). Phage DNAs from three positive clones were purified by the plate lysate method as described in section 2.5.1.2, and the genomic DNA inserts were digested with *Not* I and subcloned into the *Not* I site of pBluescript II SK+ (Stratagene).

2.8 Construction of D-enzyme expression vector and its expression in *E.coli*

2.8.1 Construction of over-expression vector

Plasmid pKK233-2 (Pharmacia) was used to construct a D-enzyme gene expression vector. A new *Nco*I site was introduced into the translational initiation sequence of pDPE-102P using an *in-vitro* mutagenesis system (United States Biochemical Corporation, Cleveland, Ohio) and a synthetic 21-mer mutagenic primer; (5'-GCGCTATCC<u>ATG</u>GTAATGAAT-3'). The resulting plasmid pDPE-102P(Nco) was digested completely with *Pst*I and partially with *Nco*I, and a 2.1 kb *Nco*I-*Pst*I fragment which covers the entire coding sequence was purified and subcloned into *Nco*I and *Pst*I sites of pKK233-2. This expression vector was designated as pKK233-DPE.

2.8.2 Purification of recombinant potato D-enzyme from *E. coli*

E.coli MC1061Δ*glg* cells carrying the plasmid pKK233-DPE were cultured in LB medium at 37 °C until late log phase, then the inducer IPTG (1 mM) was added and incubated for 6 h at 15 °C. Cells were harvested by centrifugation and disrupted by sonication in 20 mM Tris-Cl (pH 7.5) containing 5 mM 2-mercaptoethanol (buffer

A) at 4 °C. This crude extract was centrifuged and the supernatant filtered through 0.45 μm membrane, then loaded onto a Q-Sepharose fast flow column (16 x 100 mm, Pharmacia Biotech) and washed with 150 mM NaCl in buffer A. D-enzyme was eluted with buffer A containing 450 mM NaCl, and dialysed against buffer A. Concentrated Tris buffer, ammonium sulfate and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate and 5 mM 2-mercaptoethanol. The solution was loaded on to a Phenyl-TOYOPEARL 650M (TOSOH) column (10 x 100 mm) and eluted with a linear gradient of 500 to 0 mM ammonium sulfate in buffer A. Active fractions were pooled and dialysed against buffer A. The dialysate was loaded on to a Resource Q-column (6 ml, Pharmacia Biotech). The enzyme was eluted with a linear gradient of 150 to 400 mM NaCl in buffer A. Active fractions were concentrated by Amicon centricon 30 and dialysed against 20 mM sodium citrate buffer (pH 7.0).

2.9 Production of anti-D-enzyme antisera

2.9.1 Construction of expression vectors

In order to raise the antibody for potato D-enzyme, large scale preparation of purified D-enzyme polypeptide was carried out. For this purpose, expression of recombinant fusion protein of potato D-enzyme and *E. coli* glutathione transferase (GST) was attempted, since this fusion protein can be purified by single purification step using a glutathione Sepharose column. A plasmid vector pGEX-3X (Pharmacia) was employed after modification. The following oligonucleotide adapter:

5'-GATCCCCATGGAACTCGAGAAGCTTG
GGGTACCTTGAGCTCTTCGAACTTAA-5'
(*Bam*H I) *Nco* I *Xho* I *Hind* III (*Eco*R I)

was synthesised and introduced into the *Bam*H I and *Eco*R I sites of pGEX-3X and the plasmid pGEX-301, which had three additional restriction sites (*Nco* I, *Xho* I, *Hind* III), was produced.

The *Xho* I - *Hind* III fragment of pDPE102P (encoding the C-terminal 370 amino acids of D-enzyme) was introduced into the same restriction sites of pGEX-301 and pGEX-301DL produced.

2.9.2 Expression of fusion protein in *E. coli*

E.coli cells carrying the plasmid pGEX-301DL were cultured in LB medium at 37 °C until late log phase, then the inducer IPTG (final 2 mM) was added and incubated for a further 3 h at 37 °C. Cells were harvested by centrifugation and disrupted by sonication. After centrifugation both supernatant (soluble fraction) and precipitate (insoluble fraction) were analysed by SDS-PAGE. An abundant band corresponding well to the expected molecular size of the fusion protein was found only in the insoluble fraction, suggesting that the fusion protein was expressed as inclusion body in *E. coli* cells.

2.9.3 Large scale preparation of fusion protein from recombinant *E. coli*

E. coli cells, harvested from 200 ml of culture as above, were resuspended in 10 volumes of buffer B (50 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM EDTA), then lysozyme (final concentration of 1 mg ml^{-1}) was added and incubated at room temperature for 20 min. After centrifugation at 2,000 *g* for 10 min, the supernatant was discarded and the pellet resuspended in ice-cold buffer B with 0.1 % (w/v) sodium deoxycholate and incubated on ice with occasional mixing for 10 min. $MgCl_2$ and DNaseI were added to the mixture to give the final concentrations of 8 mM and 10 μg ml^{-1}, respectively, and incubated for further 30 min with occasional mixing on ice. The mixture was centrifuged at 2,000 *g* for 10 min, and the pellet was resuspended in 1.5 ml of 1 x SDS gel sample buffer. The sample was separated by SDS-PAGE, and the protein in the gel was stained in 0.01 % (w/v) Coomassie Brilliant blue in water, then the portion of the gel containing fusion protein was excised and the fusion protein was electro-eluted.

2.9.4 Immunisation of rabbits and preparation of antisera

Purified fusion protein (total 980 μg) was injected into a rabbit three times, and the rabit was bled 12 days after the third injection (done by staff of Edinburgh University Medical Faculty). Antisera were prepared by centrifugation of serum and kept at -20 °C.

2.10 Production of transgenic plants

2.10.1 Construction of plant transformation vectors and their conjugation into *Agrobacterium tumefaciens.*

Recombinant plasmids for plant transformation were constructed either in pBI101 or in pBI121 (Clontech) which contains the necessary genes and sequences for *Agrobacterium* mediated transformation. Such vectors were transformed into *E.coli* strain XL1-Blue (Stratagene). Conjugation of these plasmids into *A. tumefaciens* involved a triparental mating of the donor and host strains along with *E. coli* strain HB101 carrying a conjugation helper plasmid, pRK2013, essentially as described in Draper *et al.* (1988).

Single colonies of donor and helper strain of *E. coli* and *A. tumefaciens* LBA4404 were picked and used to inoculate 5 ml of LB medium containing 100 μg ml^{-1} kanamycin for the two *E. coli* strains and 100 μg ml^{-1} rifampicin and 300 μg ml^{-1} streptomycin for *A. tumefaciens*. The *E. coli* strains were incubated at 37 °C and *A. tumefaciens* strain at 28 °C overnight with shaking. 100 μl of each of the three cultures was pipetted onto a LB agar plate, mixed by spreading with a glass rod and incubated at 28 °C overnight. One streak of cells was removed from each plate, resuspended in 500 μl of LB medium and plated onto LB agar plates containing 100 μg ml^{-1} kanamycin, 100 μg ml^{-1} rifampicin and 300 μg ml^{-1} streptomycin. The plates were incubated at 28 °C for several days. *A. tumefaciens* cells which recieved plasmid from *E. coli* form colonies on this plate and were used for transformation of potato plants.

2.10.2 Stem cutting transformation

Internodal stem sections of *in vitro* grown shoots of potato and 100 μl of recombinant *Agrobacterium* strain overnight culture, in LB medium containing 100 μg ml^{-1} of rifampicin, 50 μg ml^{-1} of kanamycin and 200 μg ml^{-1} of streptomycin, were incubated in 20 ml of MS-medium containing 3 % (w/v) sucrose for 15 min with gentle shaking at room temperature in the dark. The infected stem pieces were then placed on callus induction solid medium and incubated for 48 h in the dark at 20 °C. These stem pieces were then transferred on to fresh callus induction solid medium containing 250 μg ml^{-1} carbenicillin. After incubation for 4 days at the same temperature but in the light, these stem pieces were transferred to fresh callus induction solid medium containing 250 μg ml^{-1} carbenicillin and 50 μg ml^{-1}

kanamycin and subcultured every 2 weeks on the same medium. Stem pieces which started to produce green healthy callus were then transferred to shooting medium containing 250 μg ml^{-1} carbenicillin and 50 μg ml^{-1} kanamycin and incubated under the same conditions. Healthy shoots appearing from the callus were rooted twice on rooting medium containing 250 μg ml^{-1} carbenicillin and 50 μg ml^{-1} kanamycin to confirm their kanamycin resistance.

2.10.3 *In vitro* shoot culture to maintain the transgenic potato plants

Transgenic potato plants were maintained as *in vitro* shoot cultures in shoot culture medium containing 250 μg ml^{-1} carbenicillin and 50 μg ml^{-1} kanamycin , and subcultured every three weeks onto fresh medium. The shoots which developed roots can be transferred to compost in small pots, and incubated in the growth cabinet. High humidity was initially maintained by covering the pots with plastic bags, which were gradually pierced to allow accustomisation to the environment and eventually removed after approximately 10 days. Such plants were grown either in growth cabinet or in glasshouse.

Chapter 3

Isolation of D-enzyme gene and its expression in *E. coli*

3.1 Introduction and aims

D-enzyme has been found in several plant organs where starch is accumulated and known to be localised in the plastid where starch is accumulated, so it is believed to be a starch metabolic enzyme. Major starch metabolic enzymes, e.g. AGPase, SS, BE and starch phosphorylase, have been purified from several plant sources, and their catalytic properties have been extensively analysed. The cDNAs or genomic DNAs for these enzymes were also isolated in most cases, and used to study their gene expression or to produce transgenic plants. Thus for these major starch metabolic enzymes, the knowledge and experimental materials have been accumulated in recent few years and their roles in starch metabolism have become relatively well understood. In the case of D-enzyme, however, neither cDNA nor genomic DNA was isolated, and even purified enzyme had not been obtained. Thus the study of D-enzyme is behind that of other major starch metabolic enzymes and no experimental result has been obtained to investigate the role of D-enzyme in starch metabolism *in vivo*.

The aim of this chapter is to establish the method to obtain pure D-enzyme for biochemical analysis and to obtain experimental materials for molecular biological analysis, such as cDNA and genomic DNA clones, and antibodies, which are necessary in the following chapters.

3.2 Purification of D-enzyme from potato tuber

Potato tuber was chosen as a source for D-enzyme, because the catalytic properties of potato D-enzyme were relatively well understood and because the system has been established in potato plants to obtain transgenic plants which will be useful in the following chapter. D-enzyme was isolated from mature potato tubers and assayed by its ability to release glucose from maltotriose. Purification employed the four chromatography procedures shown in Table 3.1 and section 2.3.1. These steps consisted of anion exchange FPLC using Q-sepharose, hydrophobic FPLC using Phenyl-Toyopearl, and two cycles of anion exchange HPLC using PL-SAX. The purified enzyme shows a single band on SDS-polyacrylamide gel electrophoresis, with an estimated molecular weight of 60,000 (Figure 3.1, lane 2). The increase in specific activity of the enzyme during purification (Table 3.1) is an underestimate, since the high total activity value in the crude extract is due to the endogenous glucose present in the potato tuber.

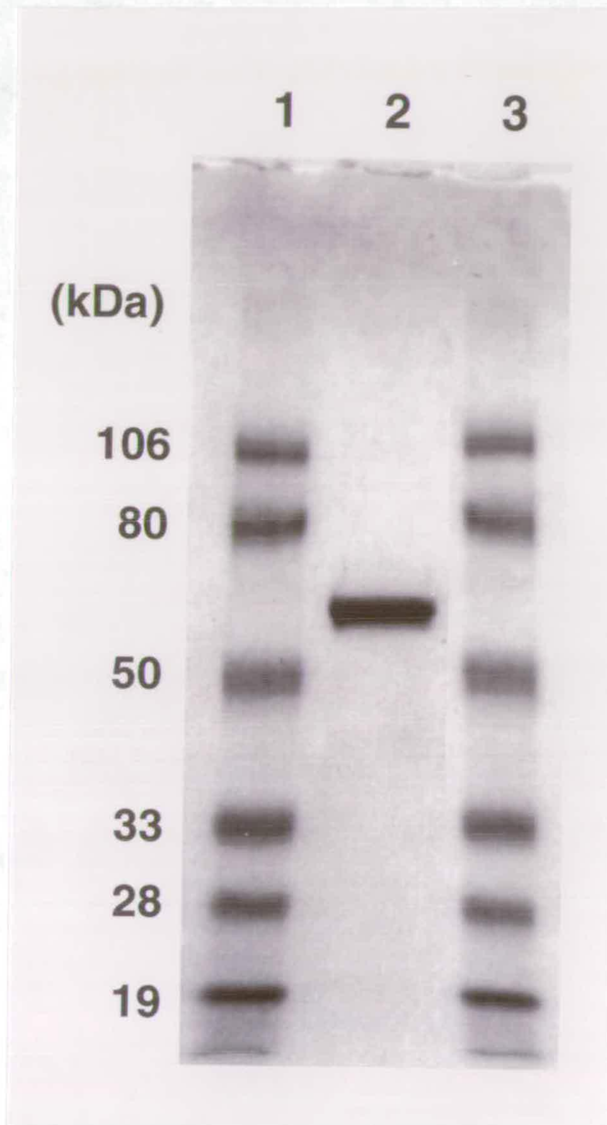


Figure 3.1 SDS-polyacrylamide gel electrophoresis of potato D-enzyme purified from tubers.
D-enzyme was purified as described in Table 3.1 from potato tubers, fractionated by SDS polyacrylamide gel electrophoresis, and stained with Coomassie Blue. Lane 1 and 3, marker proteins; Lane 2, enzyme purified from tubers. Numbers on the left are the estimated molecular weights of marker proteins, determined by the manufacturer (Bio-Rad).

Table 3.1 Purification of D-enzyme from 2.5-kg potato tubers

Step	Total activity (units)	Total protein (mg)	Specific activity (units / mg)	Activity recovered (%)
Crude extract	6202.2	4168.80	1.49	
Q-sepharose	474.7	180.00	2.64	100
Phenyl TOYOPEARL	48.6	6.27	7.75	10.2
1st PL-SAX (HPLC)	23.7	0.67	35.70	5.0
2nd PL-SAX (HPLC)	21.8	0.46	47.50	4.6

Activity recovered after each step is expressed as a percentage of activity present in the sample after the Q-sepharose step. The values are those of a typical purification.

3.3 Identification of the purified polypeptide as D-enzyme

To confirm the identity of D-enzyme, the activity of the enzyme on maltooligosaccharides was investigated. Substrates were incubated with or without enzyme for 18 h then separated by TLC. Figure 3.2 shows that no transglycosylation reaction could be detected with maltose (G2) as substrate, but that maltotriose (G3) was an effective substrate, producing other maltooligosaccharides and glucose, but not maltose. These results agree well with the reported action of D-enzyme on maltooligosaccharides, thus strongly indicating that the purified protein is D-enzyme.

3.4 Amino acid sequence analysis

N-terminal amino acid sequencing of purified D-enzyme was carried out by automated Edman degradation, revealing the first five residues (AVPAV). To obtain information about internal amino acid sequences, purified D-enzyme was digested with trypsin, and tryptic fragments were separated by reverse-phase HPLC. Two well separated peaks were collected and also subjected to amino acid sequence analysis. Sequence analysis produced amino acid sequences for both peptides (HLAALEEVYQSEK and VNYSTISEIK).

3.5 Isolation and structural analysis of cDNA clone

3.5.1 Isolation of cDNA clones for D-enzyme

A mixed (32-fold redundant) oligonucleotide probe encoding EEVYQ was synthesised (5'-TGRTANACYTCYTC-3') to use as a hybridisation probe to screen a cDNA library. cDNA was synthesised from polyA$^+$ RNA isolated from immature (1 cm) tubers and a library constructed in bacteriophage lambda vector gt10. Three positive plaques were isolated from 5 x 10^4 recombinant plaques, one of which was shown by partial nucleotide sequence analysis to encode D-enzyme. This clone was used as a probe to screen a further 2 x 10^4 plaques, and three more clones isolated. All four clones had cDNA inserts of about 2 kbp, and encoded D-enzyme as shown by partial nucleotide sequencing. Only one cDNA contained a polyA tail, but otherwise, no differences in nucleotide sequence were detected in the 3' ends of each cDNA. One clone, pDPE-102 was selected for detailed analysis.

3.5.2 Sequence analysis

The sequence of pDPE102 was determined completely, and is shown in Figure 3.3. The 5' end consists of 13 nucleotides, followed by an open reading frame of

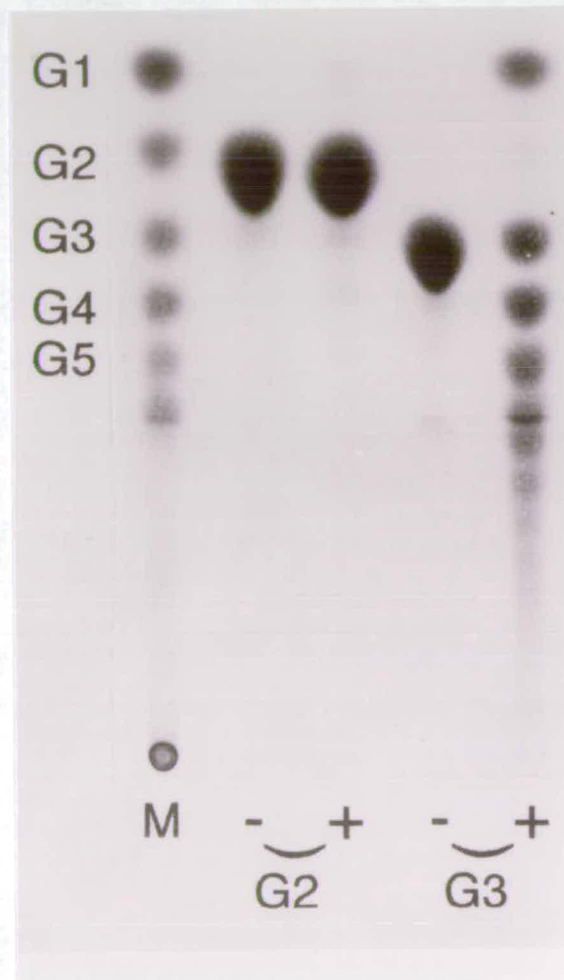


Figure 3.2 Thin layer chromatogram of reaction products from the activity of purified D-enzyme on oligosaccharides.

The reaction mixture (30 µl) containing 300 µg of maltose (G2) or maltotriose (G3), was incubated with (+) or without (-) enzyme (0.1 units) at 37 °C for 18 h. 20 µl of the reaction mixture was analysed by thin layer chromatography and stained with H_2SO_4 in methanol. M, Standard maltooligosaccharides; G2, G3, G4 and G5 are maltose, maltotriose, maltotetraose and maltopentaose, respectively.

```
   1 ATTCTCATTATCAATGGCAATTCACACTTGTTTCTCACTAATACCTTCTTCTTTCTCTTCTCCCAAATTGCCATACCCCAAAAACACTAC
                     M  A  I  H  T  C  F  S  L  I  P  S  S  F  S  S  P  K  L  P  Y  P  K  N  T  T
                                                                                        ∇
  91 TTTTCAATCTCCTATCCCAAAATTATCTAGACCCACTTTCATGTTTGATCGGAAAGGTTCCTTTCAAAATGGCACCGCTGCCGTTCCTGC
      F  Q  S  P  I  P  K  L  S  R  P  T  F  M  F  D  R  K  G  S  F  Q  N  G  T  A  A  V  P  A

 181 TGTAGGTGAGGATTTTCCGATTGATTATGCTGATTGGTTGCCAAAACGAGACCCAAACGATCGTAGAAGAGCTGGAAT TTTGCTCCATCC
      V  G  E  D  F  P  I  D  Y  A  D  W  L  P  K  R  D  P  N  D  R  R  R  A  G  I  L  L  H  P

 271 GACGTCGTTTCCTGGACCTTATGGTATTGGTGACCTTGGCCCTCAGGCTTTTAAGTTCCTTGATTGGCTTCATCTTGCTGGTTGCTCCCT
      T  S  F  P  G  P  Y  G  I  G  D  L  G  P  Q  A  F  K  F  L  D  W  L  H  L  A  G  C  S  L

 361 TTGGCAGGTTCTTCCACTTGTACCGCCTGGAAAGAGAGGCAATGAAGATGGATCACCCTATTCAGGACAGGATGCAAATTGTGGAAACAC
      W  Q  V  L  P  L  V  P  P  G  K  R  G  N  E  D  G  S  P  Y  S  G  Q  D  A  N  C  G  N  T

 451 ACTTCTGATTTCTCTTGAAGAGCTTGTTGATGATGGTTTACTGAAGATGGAGGAGCTTCCGGAGCCACTACCTACAGATCGTGTCAATTA
      L  L  I  S  L  E  E  L  V  D  D  G  L  L  K  M  E  E  L  P  E  P  L  P  T  D  R  V  N  Y

 541 CTCGACTATATCTGAGATAAAAGATCCTTTAATAACCAAGGCAGCAAAGAGGCTTCTCTCCAGTGAAGGGGAACTGAAAGACCAGCTCGA
      S  T  I  S  E  I  K  D  P  L  I  T  K  A  A  K  R  L  L  S  S  E  G  E  L  K  D  Q  L  E

 631 GAACTTTCGCCGGGATCCAAATATTTCGAGTTGGCTGGAGGATGCTGCTTATTTTGCTGCCATAGACAACTCTGTAAACACTATTAGCTG
      N  F  R  R  D  P  N  I  S  S  W  L  E  D  A  A  Y  F  A  A  I  D  N  S  V  N  T  I  S  W

 721 GTATGATTGGCCTGAACCATTGAAAAATCGCCATCTTGCAGCTCTAGAAGAAGTTTATCAAAGTGAAAAGGATTTTATTGACATATTCAT
      Y  D  W  P  E  P  L  K  N  R  H  L  A  A  L  E  E  V  Y  Q  S  E  K  D  F  I  D  I  F  I

 811 TGCACAACAGTTCTTGTTCCAACGACAATGGAAAAAAGTTCGTGACTATGCACGATCCAAAGGAATCAGTATAATGGGAGACATGCCAAT
      A  Q  Q  F  L  F  Q  R  Q  W  K  K  V  R  D  Y  A  R  S  K  G  I  S  I  M  G  D  M  P  I

 901 ATATGTTGGATATCACAGTGCTGATGTTTGGGCCAACAAGAAACAATTTTTGCTGAATAGGAAAGGTTTCCCTCTTATAGTTAGTGGTGT
      Y  V  G  Y  H  S  A  D  V  W  A  N  K  K  Q  F  L  L  N  R  K  G  F  P  L  I  V  S  G  V

 991 TCCTCCAGACGCCTTTAGTGAAACTGGTCAACTATGGGGCAGCCCTCTCTATGATTGGAAAGCCATGGAGAAGGATGGATTTTCATGGTG
      P  P  D  A  F  S  E  T  G  Q  L  W  G  S  P  L  Y  D  W  K  A  M  E  K  D  G  F  S  W  W

1081 GGTACGCCGAATTCAACGTGCAACGGATCTTTTTGATGAATTTAGGATAGATCACTTTAGAGGATTTGCTGGATTTTGGGCTGTTCCTTC
      V  R  R  I  Q  R  A  T  D  L  F  D  E  F  R  I  D  H  F  R  G  F  A  G  F  W  A  V  P  S

1171 TGAGGAAAAAATTGCAATTCTGGGACGGTGGAAGGTGGGACCTGGAAAGCCTTTGTTTGATGCTATCTTACAAGCTGTTGGGAAGATCAA
      E  E  K  I  A  I  L  G  R  W  K  V  G  P  G  K  P  L  F  D  A  I  L  Q  A  V  G  K  I  N

1261 TATTATAGCAGAAGACTTGGGAGTAATTACCGAGGACGTTGTTCAGCTAAGAAAGTCCATTGAGGCACCTGGAATGGCTGTACTCCAGTT
      I  I  A  E  D  L  G  V  I  T  E  D  V  V  Q  L  R  K  S  I  E  A  P  G  M  A  V  L  Q  F

1351 TGCATTTGGCAGTGACGCTGAAAACCCTCATTTACCTCACAATCATGAGCAGAACCAAGTAGTGTATACTGGAACACATGACAATGATAC
      A  F  G  S  D  A  E  N  P  H  L  P  H  N  H  E  Q  N  Q  V  V  Y  T  G  T  H  D  N  D  T

1441 GATCCGAGGTTGGTGGGATACTTTGCCACAGGAAGAGAAATCCAATGTACTAAAGTATTTATCAAATATTGAGGAAGAGGAAATATCACG
      I  R  G  W  W  D  T  L  P  Q  E  E  K  S  N  V  L  K  Y  L  S  N  I  E  E  E  E  I  S  R

1531 GGGCTTGATCGAAGGTGCAGTTTCTTCTGTAGCCCGTATTGCAATTATACCGATGCAGGATGTTCTTGGGCTTGGGAGTGATTCCAGAAT
      G  L  I  E  G  A  V  S  S  V  A  R  I  A  I  I  P  M  Q  D  V  L  G  L  G  S  D  S  R  M

1621 GAACATTCCAGCAACTCAGTTTGGAAACTGGAGTTGGAGGATACCTAGTTCAACTAGCTTTGACAACCTGGATGCAGAAGCAAAAAAGCT
      N  I  P  A  T  Q  F  G  N  W  S  W  R  I  P  S  S  T  S  F  D  N  L  D  A  E  A  K  K  L

1711 AAGAGATATACTTGCAACTTATGGGCGGTTGTGATGATGAATGATGATTAAGATGTTATGTCCCCTGGGAATCAGGGGAGGACCTAGGCT
      R  D  I  L  A  T  Y  G  R  L

1801 GTAAGGACGTTCTCAGGCTTGGGACACAAATTGCTGAGAGCATAAACGATCTAAACAAGGTTTTCCTGCCACAGTTCACCTCATTGCTGC

1891 AAAGGAACGCCTGCATTTTCAATTTTCTGAACTTTAGGCTCACTGGGGAAGCAGACAAGGAAAGCTAAAACGCGTGCTTTGGATTCTATT

1981 ATTCTACATTAGCTGGTATAAACTACGAATGCTAGAGCCTTTCCATATTTATGCAGAAAGACTTCATAGTTTGAATGTCACCTGACCTAA

2071 AAATAAATAATGCATGTTTTTGTATTTACTATTTGGTCG
```

Figure 3.3 Nucleotide and deduced amino acid sequences of D-enzyme cDNA

The DNA sequence of pDPE102 was determined completely on both strands. The N-terminal end of the mature protein is marked by an arrow. Peptide sequences derived from the purified enzyme are underlined. A putative polyadenylation signal is doubly underlined.

1731 nucleotides, a 3' untranslated sequence of 368 nucleotides, but no polyA tail. The cDNA clone with the polyA tail had a 3' untranslated region which was about ten nucleotides longer than pDPE102 (the polyA interfered with nucleotide sequencing). This observation suggests that the sequence 5' AATAAA 3' underlined in Fig. 3.3, (position 2072) could be a polyadenylation signal (Proudfoot and Brownlee, 1976). The sequence around the putative translation initiation site 5' ATCAATGGC 3' is similar to a proposed plant mRNA consensus, 5' AACAATGGC 3' (Luetcke *et al.*, 1987). The open reading frame encodes a polypeptide of 577 amino acid residues (M_r 64,950), but the amino acid sequence found at the N-terminus of the purified enzyme (arrowed in Fig. 3.3) starts at amino acid position 53. It is deduced that the first 52 amino acid residues constitute a transit peptide for targeting to the plastid. The remaining 525 residues constitute the mature enzyme (M_r 59,500). The two tryptic peptides found in the enzyme are underlined in Fig. 3.3, helping to confirm the identity of the cDNA clone.

3.5.3 Homology analysis

The identity is further confirmed by comparison of the deduced sequence of the potato enzyme with four bacterial amylomaltase (α-1,4-glucanotransferase), sequences which are available in the data base. The summary of homology analysis is shown in Figure 3.4. Potato D-enzyme shows significant homology to amylomaltases from *Clostridium butyricum* (40.2 % identity) and from *Streptococcus pneumoniae* (36.6 % identity), but less homology to those from *Escherichia coli* (11.6 % identity) and *Haemophilus influenzae* (10.2 % identity). The alignment of five genes showed that these 5 enzymes may be divided into two subgroups, one group consists of potato, *S. pneumoniae* and *C. butyricum* enzymes and the other consists of *E. coli* and *H. influenzae* enzymes, because the similarity is very high within each subgroup, but less between subgroups (Figure 3.4 and 3.5). However, five regions (A to D) which are highly conserved in all five enzymes were found. These similarities found in primary sequence strongly indicated that the cDNA clone encodes D-enzyme.

3.5.4 Expression in *E. coli*

Expression of the cDNA clone in *E. coli* was sought in order to obtain final confirmation of its identity. The plasmid vector pKK233-2 containing a *trc* promoter (with 17 base pairs between the *trp* -35 region and the *lac* UV5 -10 region) was employed to direct expression of the complete cDNA open reading frame (see section 2.8). Following induction of gene expression with IPTG at 37 °C, D-enzyme activity

	Potato	*C. but.*	*S. pnu.*	*E. coli*	*H. inf.*
Potato	100	40.2	36.6	11.6	10.2
C. but.	-	100	48.1	13.2	11.0
S. pnu.	-	-	100	13.7	12.1
E. coli	-	-	-	100	36.9
H. inf.	-	-	-	-	100

Figure 3.4 Summary of identity of D-enzyme with bacterial amylomaltases
The deduced amino acid sequence of potato D-enzyme (Potato) was compared with bacterial amylomaltase from *Clostridium butyricum* (*C. but.*), *Streptococcus pneumoniae* (*S. pne.*), *Escherichia coli* (*E. coli*) and *Haemophilus influenzae* (*H. inf.*) by the GENETYX program (Software Development Co., Ltd., Tokyo, Japan). Values show percent amino acid sequence identity. Genbank accession numbers for enzymes from *C. butyricum*, *S. pneumoniae* , *E. coli* and *H. influenzae* are L37874, J01796, M32793 and U32760, respectively.

```
      HINF.PTN    1 : VVSMQISDSLKQKAEKCGIALSHYDIDGHLIFADEKTVSTFVELLQPPPK
  ECOMALQP.PTN    1 :      MESKRLDNAALAAGISPNYINPHGKPQSISAETKRRLLDAMHQRTA

  STDPEPA.PTN     1 :                          MAIHTCFSLIPSSFSSPKLPYPKNTTF
      HINF.PTN   51 : AKGQFDDVLAAFENEPIDYRLNRLDLPPSAEYRYQLTDESNAILLEKILS
  ECOMALQP.PTN   47 : TKVAVTPVPNVMVYTS-GKKM-PMVVEGSGEYSWLLTTEEGTQYKGHVTG

  STDPEPA.PTN    28 : QSPIPKLSRPTFMFDRKGSFQNGTAAVPAVGEDFPIDYADWLPKRDPNDR
  STRMALMX.PTN    1 :                                                MKK
      HINF.PTN  101 : NLSVLSLPP-LPFGYYQLSIFSDTEQYRIRLLISPKTAFQP-PVLENKKV
  ECOMALQP.PTN   97 : G-KAFNLPTKLPEGYHTLTLTQDDQRAHCRVIVAPKRCYEPQALLNKQKL

  STDPEPA.PTN    78 : RRAGILLHPTSFPGPYGIGDLGPQAFKFLDWLHLAGCSLWQVLPL-----
  STRMALMX.PTN    4 : RQSGVLMHISSLPGAYGIGSFGQSAYDFVDFLVRTKQRYWQILPL-----
  CLOMALQP.PTN    1 :        MHISSLPGKYGIGTFGRSAYEFCDFLEKAGQKYWQILPL-----
      HINF.PTN  151 : WGVNVQLYSLRSEQNWGIGDFGDLAYLIEQSAKQGADYVGINPLHLPYPA
  ECOMALQP.PTN  147 : WGACVQLYTLRSEKNWGIGDFGDLKAMLVDVAKRGGSFIGLNPIHALYPA
                                  ---------
                                  Region A

  STDPEPA.PTN   128 : ------VPPGKRGNEDGSPYSGQDANCGNTLLISLEELVDDGLLKMEELP
  STRMALMX.PTN   54 : ---------GATSYGD-SPYQSFSAFAGNTHFIDLDILVEQGLLEASDLE
  CLOMALQP.PTN   45 : ---------GQTSYGD-SPYQSFSAFAGNPYFIDLDILNEKNLLDKDDYE
      HINF.PTN  201 : VPNWASPYSSSSRRWLNFLYLDIPDLPEFKRCRSVQNWFKREDIQAKIAA
  ECOMALQP.PTN  197 : NPESATAYSPSSRRWLNVIYIDVNAVEDFHLSEEAQAWWQLPTTQQTLQQ

  STDPEPA.PTN   178 : E-PLPTD--RVNYSTISEIKDPLITKAAKRLLSSEGELKDQLENFRRDPN
  STRMALMX.PTN  104 : GVDFGSDASEVDYAKIYYARRPLLEKAVKRFFEV-GDVKD-FEKFAQD--
  CLOMALQP.PTN   95 : EKNFGDNKEMINYGLIFNEKMKVLRKAYMNFNSKDD---ESFAKFIED--
      HINF.PTN  251 : LRESDCVDYSSILALKLTALEPLFDFFQRSQSVEIVTRRKIFAEYLKNKG
  ECOMALQP.PTN  247 : ARDADWVDYSTVTALKMTALRMAWKGFAQRDDEQMAAFR----QFVAEQG

  STDPEPA.PTN   228 : ISSWLEDAAYFAAIDNSVNTISWYDWPEP-LKNRHLAALEEVYQSEKDFI
  STRMALMX.PTN  154 : NQSWLELFAEYMAIKEYFDNLAWTEWPDADARARKASALESYREQLADKL
  CLOMALQP.PTN  145 : EKKWLDDYSLFMALKYKFNFISWNSW-NKDIKLRKNEEIEKYKDELKEDV
      HINF.PTN  301 : EPLLLQGLFNVLDLQEHADHQAEENTIGWLGWRKEWQHLSAAKRKALLKT
  ECOMALQP.PTN  297 : DSLFWQAAFD--ALHAQQVKE-DEMRWGWPAWPEMYQNVDSPEVRQFCEE

  STDPEPA.PTN   278 : DIFIAQQFLFQRQWKKVRDYARSKGISIMGDMPIYVGYHSADVWANKKQF
  STRMALMX.PTN  204 : VYHRVTQYFFFQQWLKLKAYANDNHIEIVGDMPIYVAEDSSDMWANPHLF
  CLOMALQP.PTN  195 : NYWKFLQYEFFSQWKNLKDYANKKNIKIIGDIPIYIAQDSSDVWSNPDIF
      HINF.PTN  351 : HHEKIQFFAWLQWLTEEQLSALQNLCKQSGMKLGIYGDLAVNSSRGSADV
  ECOMALQP.PTN  347 : HRDDVDFYLWLQWLAYSQFAACWEISQGYEMPIGLYRDLAVGVGTGGAET

  STDPEPA.PTN   328 : LLNRKG-FPLIVSGVPPDAFSETGQLWGSPLYDWKAMEKDGFSWWVRRIQ
  STRMALMX.PTN  254 : KTDVNG-KATCIAGCPPDEFSVTGQLWGNPIYDWEAMDKDGYKWWIERLR
  CLOMALQP.PTN  245 : LLNKETLEPLKVSGCPPDAFSETGQLWGNPIYDWGYLEKTNFEWWVDRIK
      HINF.PTN  401 : HSDPDLYCVNASIGAPPDPLGPVGQNWNLPPYNPTVLKARGFAPFIDMLC
  ECOMALQP.PTN  397 : HCDRELYCLKASVGAPPDILGPLGQNWGLPPMDPHIITARAYEPFIELLR
                                 -----------------
                                     Region B

  STDPEPA.PTN   378 : RATDLFDEFRIDHFRGFAGFWAVPSEEKIAILGRWKVGPGKPLFDAILQA
  STRMALMX.PTN  304 : ESFKIYDIVRIDHFRGFESYWEIPAGSDTAAPGEWVKGPGYKLFAAVKEE
  CLOMALQP.PTN  295 : SSLKLYDILRIDHFRGFEAYWSVDYGEKTAQNGKWIKGPEMKLFNVIKEK
      HINF.PTN  451 : ANRQYFGVLRIDHVMGLFRLWVIPKGKTAADGAYVHYPFDELMAILAIES
  ECOMALQP.PTN  447 : ANRQNCGALRIDHVMSMLRLWWIPYRETADQGAYVHYPVDDLLSILALES
                             ------------
                               Region C

  STDPEPA.PTN   428 : VGKIN-IIPEDLGVITEDVVQLRKSIEAPGMAVLQFAFGSDAENPHLPHN
  STRMALMX.PTN  354 : LGELN-IIAEDLGFMTDEVIELRERTGFPGMKILQFAFNPEDESIDSPHL
  CLOMALQP.PTN  345 : IGDIE-IIAEDLGYLTEETLEFKKRTGFPGMKILQFAFGGDSSNPYLPHN
      HINF.PTN  501 : VRNECLIIGEDLGTVPDEVRWKLNEFQIFSYFVLYFA--QRNGEFPRISD
  ECOMALQP.PTN  497 : KRHRCMVIGEDLGTVPVEIVGKLRSSGVYSYKVLYFE-NDHEKTFRAPKA
                           ------
                          Region D

  STDPEPA.PTN   478 : HEQNQVVYTGTHDNDTIRGW-WDTLPQEEKSNVLKYLSNIEEHEISRGLI
  STRMALMX.PTN  404 : APANSVMYTGTHDNNTVLGW-YRNEIDDATREYMARYTNRKEYETVVHAML
  CLOMALQP.PTN  395 : YEKNCVAYTGTHDNDTVRGW-FEVTGSKEEKEKAVEYFKLTEEEGYNWGVI
      HINF.PTN  551 : YPRNAYATIGTHDVPSLQSFWHCRDLELFNQLGILNGE-VLKQKYDQRVM
  ECOMALQP.PTN  547 : YPEQSMAVAATHDLPTLRGYWECGDLTLGKTLGLYPDEVVLRGLYQDREL
                                 ---
                               Region E

  STDPEPA.PTN   528 : EGAVSSVARIAIIPMQDVLGLGSDSRMNIPATQFGNWSWRIPSSTSFDNL
  STRMALMX.PTN  454 : RTVFSSVSFMAIATMQDLLELDEAARMNFPSTLGGNWSWRMTEDQLTPAV
  CLOMALQP.PTN  445 : RGVWSSVANTSIGVMQDFLNLGNEARINKPSTLASNWSWRAKDNVFTNEL
      HINF.PTN  601 : DKQALLNSLHRDNYLPPHYEGDALSHAMHDYLNRMIHYYLAESNSRLIGV
  ECOMALQP.PTN  597 : AKQGLLDALHKYGCLPKRAGHKASLMSMTPTLNRGLQRYIADSNSALLGL

  STDPEPA.PTN   578 : DAEAKKLRDIILATYGRL
  STRMALMX.PTN  504 : EEGLLDLTTIYRRINENLVDLKK
  CLOMALQP.PTN  495 : ANKIYRLTRIYGRCE
      HINF.PTN  651 : QLENLLSQEISFHLPSTSNEYPNWCKKLAQPLAFIFSNEALKTFFVQINQ
  ECOMALQP.PTN  647 : QPEDWLDMAEPVNIPGTSYQYKNWRRKLSATLESMFADDGVNKLLKDLDR

      HINF.PTN  701 : GRNV
  ECOMALQP.PTN  697 : RRRAAAKKK
```

Figure 3.5 Alignment of potato D-enzyme with bacterial amylomaltases

The deduced amino acid sequence of potato D-enzyme (Potato) was aligned with bacterial amylomaltase from *Clostridium butyricum* (*C. but.*), *Streptococcus pneumoniae* (*S. pne.*), *Escherichia coli* (*E. coli*) and *Haemophilus influenzae* (*H. inf.*). Black boxes indicate identical amino acids in all five sequences and hatched boxes indicate conserved amino acids within either subgroup. Highly conserved regions A to E described in the text are underlined.

was measured in extracts of *E. coli* TG-1 cells containing either the plasmid vector or recombinant plasmid, pKK233-DPE. No activity was detected in either cells. However, when cells were grown at reduced temperature following IPTG induction, significant D-enzyme activity was detected in extracts of cells containing the recombinant plasmid, but not in controls (Figure 3.6). The enzyme synthesised by cells at 15 °C was purified (using the methods described in section 2.8) and shown to have a molecular weight of approximately 65,000 (Figure 3.7). This molecular weight corresponds with that of the D-enzyme precursor (M_r 65,000) but not with that of endogenous amylomaltase of *E. coli* (M_r 78,360, Pugsley and Dubrevil (1988). The specific activity of the purified recombinant D-enzyme was the same as that of the purified D-enzyme from potato tubers. The successful synthesis of biologically active recombinant proteins in *E. coli* at reduced temperatures has been previously reported (Mori *et al.*, 1991) but we do not know its cause. However, these experiments successfully established the identity of the D-enzyme cDNA clone.

3.6 Features of the D-enzyme polypeptide

3.6.1 Function of N-terminal extension sequence

The native enzyme from potato tubers has a molecular mass of 59,500. This enzyme is apparently made as a higher molecular weight precursor of 65,000. This is entirely consistent with the observation that the enzyme is found in extracts of chloroplasts from *Pisum sativum* (Kakefuda *et al.*, 1986), spinach (Okita *et al.*, 1979) and *Arabidopsis* leaves (Lin *et al.*, 1988c). In the latter case D-enzyme was found exclusively in the chloroplast fraction. It is proposed therefore that the N-terminal amino acid pre-piece functions as a transit peptide for plastid targeting, although a chloroplast or amyloplast location for potato D-enzyme has not yet been reported. The putative transit peptide has many features in common with known transit peptides, including a high proportion of hydroxyl and basic amino acids, few acidic amino acids, and similarity to a consensus motif at the processing site (Gavel and von-Heijne, 1990). In the potato tuber the protein is presumably targeted to the amyloplast. It is not known if a single D-enzyme is synthesised throughout the potato plant, or if there are isoforms which might be specific to particular organs. However, it is well established that transit peptides can function interchangeably between chloroplast and amyloplasts (de-Boer *et al.*, 1988; Klösgen *et al.*, 1989; Klösgen and Weil, 1991) so a single D-enzyme is possible. Preliminary analysis of four tuber cDNA clones found no evidence for sequence heterogeneity.

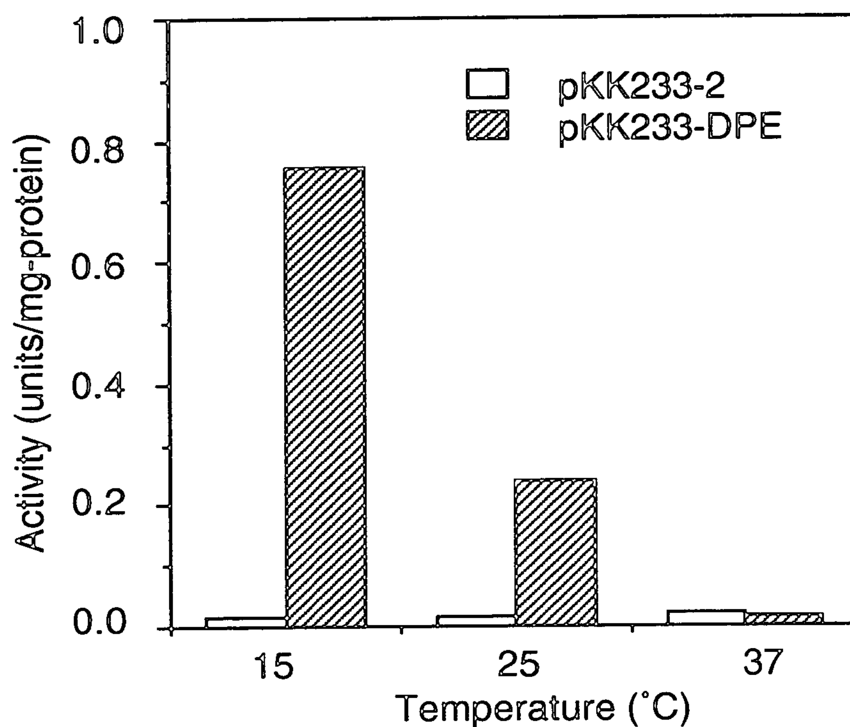


Figure 3.6 D-enzyme activity of the extract of recombinant *E. coli*
E. coli TG-1 cells transformed with pKK233-2 and pKK233-DPE were cultured with the inducer IPTG for 6 h at the indicated temperatures. Extracts prepared by sonication and centrifugation were used to assay for D-enzyme activity. The values are those of a typical experiment.

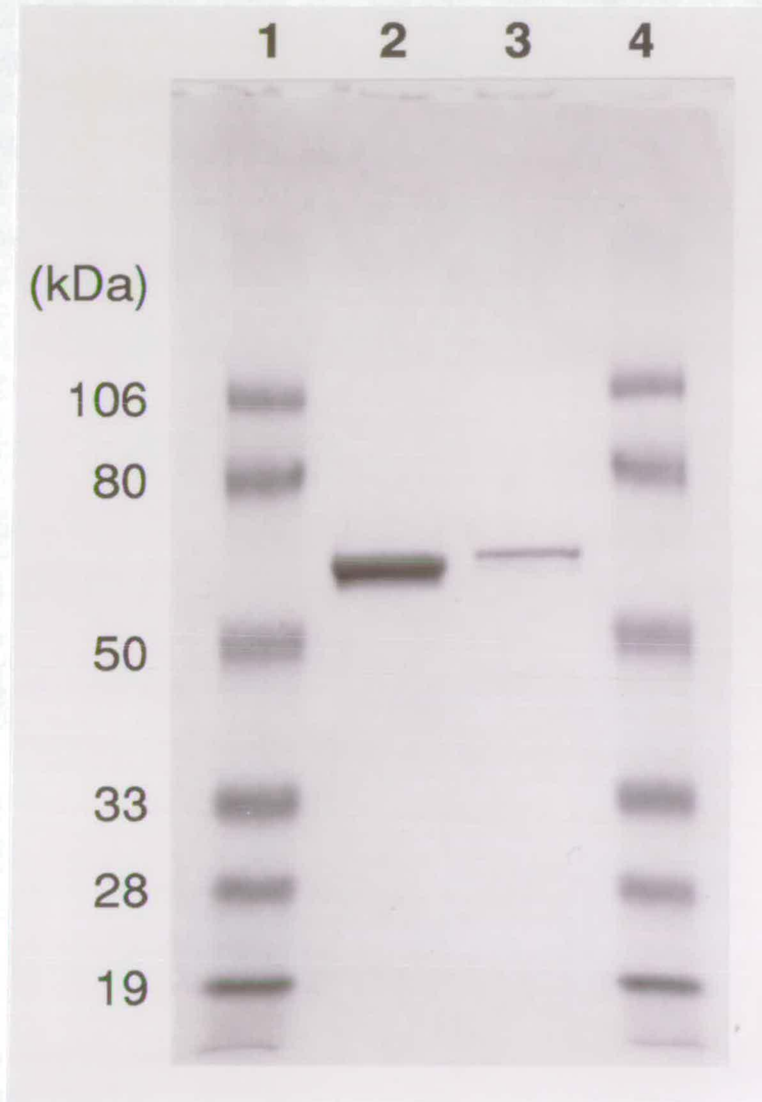


Figure 3.7 SDS-polyacrylamide gel electrophoresis of potato D-enzyme purified from tubers and recombinant *E. coli*.
D-enzyme was purified as described in Table I from potato tubers and recombinant *E. coli*, fractionated by SDS polyacrylamide gel electrophoresis, and stained with Coomassie Blue. Lanes 1 and 4, marker proteins; Lane 2, enzyme purified from tubers; Lane 3, enzyme purified from *E. coli*. Numbers on the left are the estimated molecular mass of marker proteins, determined by the manufacturer (Bio-Rad).

3.6.2 Comparison of D-enzyme with starch and glycogen metabolic enzymes

Four conserved regions have been reported to be present in enzymes which catalyse hydrolytic or transglycosidic reactions on starch (Takata *et al*., 1992). These enzymes consist of amylases, cyclodextrin glucanotransferases (CGTases), debranching enzymes and branching enzymes, and it has been proposed to call this the α-amylase family of enzymes (Takata *et al*., 1992). X-ray crystallographic structural analysis and protein engineering studies demonstrated that amino acid residues in these four conserved regions play important roles in their catalytic mechanism, which is believed to be common among this enzyme family (for review, see Matsuura, 1995). D-enzyme catalyses the glucan transfer of maltooligosaccharides, which is also catalysed by CGTase, so might be included in the α-amylase family of enzymes and may contain similar conserved amino acid sequence motifs. Interestingly, homologous regions C, D, and E found in D-enzyme/amylomaltase primary sequences showed similarity to the conserved regions 2 to 4 found in the α-amylase family of enzymes, respectively (Figure 3.8). In particular, three acidic amino acid residues, D206, E230 and D297, which are recognised as the active site in *Aspergillus oryzae* α-amylase by X-ray crystallographic structural analysis (Matsuura *et al*., 1984; Matsuura *et al*., 1991) and found in region 2 to 4, are found in all D-enzyme/amylomaltases. However, amino acid residues which are recognised as substrate binding sites are not always found in D-enzyme/amylomaltase, and a conserved region 1 homologue could not be found in D-enzyme/amylomaltase sequences. Thus the catalytic mechanisms proposed in α-amylase family enzymes may not be fully applied to D-enzyme action.

CGTase and D-enzyme resemble each other in their catalytic activity, since both enzymes catalyse the transfer of α-1,4-glucan units from one maltooligosaccharide to another or to glucose. However these two enzymes were classified into different groups, because CGTase catalyses the cyclisation reaction on starch to produce cyclodextrins, cyclic α-1,4-glucans with DP of 6 to 8, which are not produced by D-enzyme. Computer aided homology analysis between D-enzyme and several CGTases was carried out to find any similarity in their primary sequences, but no further similarity other than the previously described three highly conserved regions 2 to 4, was observed between them (Figure 3.8).

	Region1		Region2		Region3		Region4	
Amylases								
A.ory.	117	DVVANH	202	GLRIDTVKH	230	EVLD	292	FVENHD
B.amylo.	98	DVVFDH	227	GFRLDAVKH	261	EYWQ	323	FVENHD
Human,p	99	DAVINH	196	GFRLDASKH	236	EVID	298	FVDNHD
Barley	101	DIVINH	127	DFRLDWGPH	218	EVWD	299	FVDNHD
Debranching enzymes								
P.amylo.	291	DVVYNH	370	GFRFDLASV	454	EWSV	502	FIDVHD
K.aero.	600	DVVYNH	671	GFRFDLMGY	704	EGWD	827	YVSKHD
Cyclodextrin glucanotransferases								
K.pne.	130	DYADNH	219	AIRIDAIKH	257	EWFG	328	FMDNHD
B.mace.	135	DFAPNH	225	GIRFDAVKH	258	EWFL	324	FIDNHD
B.stearo.	131	DFAPNH	221	GIRMDAVKH	253	EWFL	319	FIDNHD
Branching enzymes								
E.coli	335	DWVPGH	401	ALRVDAVAS	426	EFGG	521	LPLSHD
S.sp.	370	DWVPGH	436	GIRVDAVAS	461	EYGG	556	LALSHD
Maize	277	DVVHSH	347	GFRFDGVTS	406	EDVS	470	YAESHD
D-enzyme / amylomaltase								
Potato			385	EFRIDHFRG	436	EDLG	485	YTGTHD
S.pne.			311	IVRIDHFRG	362	EDLG	411	YTGTHD
C.but.			303	ILRIDHFRG	353	EDLG	402	YTGTHD
H.inf.			458	VLRIDHVMS	510	EDLG	558	TIGTHD
E.coli			454	ALRIDHVMS	506	EDLG	554	VAATHD

Figure 3.8 Alignment of highly conserved region found in α-amylase family enzymes

Black boxes and hatched boxes in *Aspergillus oryzae* sequence are active sites and substrate-binding sites, respectively, proposed by Matsuura *et al.* (1984) for this enzyme. Putative active sites and substrate-binding sites which are also conserved in other aligned sequences are boxed as above. Abbreviations: *A. ory., Aspergillus oryzae; B. amylo., Bacillus amyloliquefaciens;* Human, P, Human pancreas; *P. amylo., Pseudomonas amyloderamosa; K. aero., Klebsiella aerogenes; K. pne., Klebsiella pneumoniae; B. mace., Bacillus macerans; B. stearo., Bacillus stearothermophilus; E. coli, Escherichia coli; S. sp., Synechococcus sp.; S. pne., Streptococcus pneumoniae; C. but., Clostridium butyricum; H. inf., Haemophilus influenzae .*

3.7 Production of antibody for D-enzyme

The cDNA clone was further used to prepare antibody for potato D-enzyme, since this is a powerful tool either to characterise the expression of this gene at the protein level or to analyse transgenic plants in the following chapters. A partial cDNA sequence was translationaly fused to a bacterial glutathione S-transferase (GST) gene as described in section 2.9, and the resultant vector pGEX-301DPE was introduced into *E. coli* XL-1 Blue cells. Following induction of gene expression with IPTG, fusion protein (63 kDa) was successfully over-expressed and accumulated in the cells as insoluble inclusion bodies. The fusion protein was solubilised from inclusion bodies and further purified by SDS-PAGE then by electroelution. The purified fusion protein was injected three times (10 to 12 day intervals) into two rabbits and antisera were prepared ten days after the final immunisation. The specificity of antisera thus obtained was checked by western blot analysis against potato tuber crude extracts, where a 60 kDa polypeptide (D-enzyme) was specifically detected. Note that when the antisera were replaced with pre-immune sera, this band was not detected.

3.8 Isolation and structural analysis of genomic DNA clone

3.8.1 Isolation of genomic DNA clones for D-enzyme

A potato genomic DNA library was constructed from leaf DNA which was partially digested with *Sau* 3AI and size fractionated by sucrose density gradient ultra-centrifugation as described in section 2.7.3. It was plated and screened for clones carrying genes for D-enzyme using the radio labelled full length cDNA. Three positive plaques were isolated, and their insert DNAs were analysed with restriction enzymes. Structures of the three clones and the cDNA clone are shown in Figure 3.9. The correspondence of three restriction sites (*Xba* I, *Eco* RV and *Eco* RI) on the genomic clones and on the cDNA clone were confirmed by partial sequencing analysis from each site.

3.8.2 Partial sequencing analysis of 5' untranslated region of the genomic DNA

In order to sequence the putative promoter region of λGDPE-1, a deletion series was generated from the *Xba* I site by using the Exo III/S1 nuclease system and nucleotide sequence was determined using the Sanger dideoxy chain termination procedure. The sequence is shown in Figure 3.10. The partial nucleotide sequence of the genomic DNA clone, in both 5' untranslated region and coding region, completely

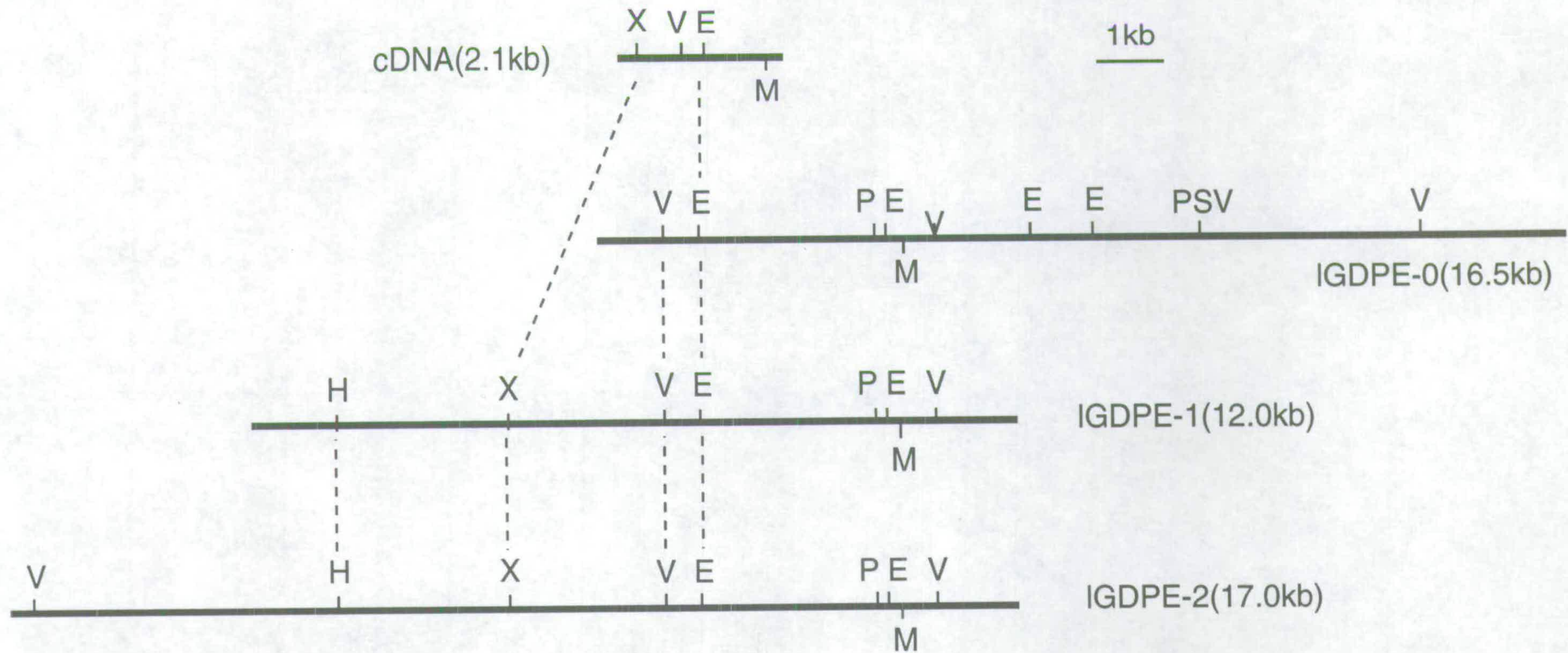


Figure 3.9 Restriction endonuclease maps of three genomic clones
Three genomic DNA fragments for D-enzyme are aligned with the cDNA. E, *Eco* RI; V, *Eco* RV; P *Pst* I; S, *Sac* I; M, *Mlu* I; X, *Xba* I; H, *Hind* III.

```
   1  AAAAAGAAATTAAGAGGAATAGAGTTATTACTTTATTTTTTAATGTTTTGTATTATGTGA

  61  AGTTGTGAACTGAAAGTCACTTTAAAGTGTGAATTGAAGGCTAGAGAACAAAGATAGTAA

 121  CTAGTAAGATATTGAGATTAATATTTAATATTTATGTACGGGTAAAGTAGTAAATTATAC

 181  ACTGTCAATGGGTTATCGGTTTACCCAATAACCCAATAATTTATTTTTAAAAAATCACTA

 241  AGAATTGTAATCCAATAGCCCAATAACGATAAACCAATGGTAATTTTTCGGTTCGATTTT

 301  TGCACACCCCCTAGACTGACACCATTTAGATCGCTTGGTAGGGTATACAAAAATAATATT

 361  TAGATATGTTGTGTTTGTATTAGTTATGCAGAAATTAATTTTTAATCATTGTTTGATTTG

 421  ATGTATTAAAAACATGCATTCTATAATATTTTTTTTTTTAAAAAAGATTTATTATAAGAA

 481  TACCTTCCAAATATGGTGAAAAATGTAAAAAAAAGTTTGAGGGCAATTATATTTTTAACC

 541  ATACTAATGCATGCATTAAAAATCTTTGTATTACTAATATATGGTTTTTCATGTATTTAT

 601  AAGTATTTTCTTATTGATTCCGAAACCAAACCATGAAGGAACCAAATCGATAAAAAATAT

 661  CTTATTGATTTGATTATTAGTTTAGCGTATTTAAAAAACTAAAAAAATAATAAATCGAGC

 721  CAATAATACATAAAATGAATTTAGCGTATTTAAAAAATTAAAATATTAATAAATCGAGCC

 781  AATAATACATAAAATCGACCCGAATCGATGTATCACATGCTTCTTGTCTCCCCACCGCAA

 841  CACCTTCCTCAACTTCCTACTCCACTCCACTCCACTGTGTTCCTTTCCATTTTCAAGCCA

 901  AAAACGGCAATCCCAATTTCAAAATCGACCCATTTTCTTCAAGCCAAATTCTCCTCCAAA

                      ▼                             ▼          ▼
 961  TCAATAATATGAAGGAAAAATACTAACAATCTTCACGTATTCTCATTATCAATGGCAATT
                                                         M  A  I
         ▼
1021  CACACTTGTTTCTCACTAATACCTTCTTCTTTCTCTTCTCCCAAATTGCCATACCCCAAA
      H  T  C  F  S  L  I  P  S  S  F  S  S  P  K  L  P  Y  P  K

1081  AACACTACTTTTCAATCTCCTATCCCAAAATTATCTAGA
      N  T  T  F  Q  S  P  I  P  K  L  S  R
```

Figure 3.10 DNA sequence of a putative promoter region
The DNA sequence of the putative promoter region of genomic DNA clone pGDPE1 was determined. Translational initiation codon is doubly underlined. The 5' sites where four cDNA clones start are indicated with solid arrow heads. One direct repeat is underlined. Sequences homologous to consensus TATA and CAT sequences are boxed.

matched the nucleotide sequence information from four cDNA clones. Thus this genomic DNA clone pGDPE1 seems to be the gene for the isolated cDNA clones. One set of direct repeats, and several TATA and CAT like sequences were found in this region and are indicated in Figure 3.10. However further analysis, eg. primer extension analysis is necessary to describe the features of promoter structure.

3.9 Conclusion

D-enzyme was purified from potato tubers using four chromatography procedures. The purified enzyme shows a single band on SDS-PAGE with an estimated molecular weight of 60,000. A cDNA clone encoding the enzyme was isolated using oligonucleotide probes derived from partial peptide sequences of the purified enzyme. The identity of the cDNA clone was confirmed by expression in *E. coli* resulting in D-enzyme activity. The amino acid sequence deduced from the cDNA shows significant homology with 4-α-glucanotransferases from bacteria. The deduced sequence indicates the presence of an amino terminal plastid transit peptide of 52 amino acid residues and a mature polypeptide of 525 residues. Recombinant GST-D-enzyme fusion protein was purified from *E. coli* cells and used to raise antibody for D-enzyme. Genomic DNA clones were isolated and their structures were characterised by restriction mapping and partial DNA sequencing. Purified enzyme, genomic DNA clone , cDNA clone and antibodies obtained in this chapter enable studies described in following chapters to be undertaken.

Chapter 4

Analysis of the action of D-enzyme

4.1 Introduction and aim

D-enzyme has been partially purified from potato tubers (Jones and Whelan, 1969), germinating barley seeds (Yoshio *et al.*, 1986) and *Arabidopsis* leaves (Lin and Preiss, 1988), and its activity on maltooligosaccharides was investigated. These studies demonstrated that D-enzyme catalyses a disproportionation reaction on maltooligosaccharides *in vitro* in which a glucan moiety is transferred from one α-1,4-glucan molecule to another, or to glucose. Maltooligosaccharides have been shown to be effective donors, maltooligosaccharides or glucose to serve as acceptors, and a maltosyl unit to be preferentially transferred (Yoshio *et al.*, 1986; Lin and Preiss, 1988). It was also demonstrated that the smallest donor molecule is maltotriose, the smallest acceptor molecule is glucose, and the major transferred glucan chain is a maltose unit, but glucosyl transfer never occurs. Based on this action of D-enzyme on small oligosaccharides, it has been proposed that the enzyme may be involved in starch breakdown to convert short maltooligosaccharides into longer maltooligosaccharides upon which starch phosphorylase can act (Lee and Whelan, 1971; Lin and Preiss, 1988).

However, as described in chapter 1, there is a possibility that high molecular weight starch might be the real substrate of the enzyme *in vivo*, rather than maltooligosaccharides. In this case we should consider other possible roles for the enzyme. It is already suggested that partially purified D-enzyme can catalyse the transfer of a maltooligosaccharide unit from the non-reducing end of amylopectin (Lin and Preiss, 1988) or soluble starch (Yoshio *et al.*, 1986) to glucose, and produce maltooligosaccharides, so the enzyme may be able to use these high molecular weight starch molecules as donors. Moreover, it is not known if the enzyme can use high molecular weight starch as an acceptor molecule. Thus, it seems to be very important to examine the action of D-enzyme on high molecular weight starch using pure enzyme.

Successful expression of D-enzyme cDNA in *E. coli* described in the previous chapter allows preparation of D-enzyme which is free from hydrolytic activity and shows a single band on SDS-PAGE. The aim of this chapter is to characterise the *in vitro* action of purified D-enzyme on maltooligosaccharides and especially on high molecular weight starch in order to obtain a better understanding of the role of D-enzyme in starch metabolism.

4.2 Action of D-enzyme on maltooligosaccharides

D-enzyme was purified from recombinant *E. coli* as described in section 3.5.4 but *E. coli* MC1061Δ*glg* (Takata *et al.*, 1994) was employed as a host strain. In this *E. coli* strain, the chromosomal region which encodes glycogen branching enzyme, debranching enzyme, glycogen synthase, AGPase and glycogen phosphorylase, were replaced by the kanamycin resistance gene from Tn903, so could avoid the contamination of endogenous branching enzyme or phosphorylase activity into the D-enzyme preparation. The absence of amylase, branching enzyme and phosphorylase activity in the final D-enzyme preparation was confirmed by assaying those enzyme activities (results not shown).

The activity of D-enzyme on a range of maltooligosaccharides was first investigated. Substrates were incubated with or without enzyme for 18 h then separated by TLC. Figure 4.1 shows that no transglycosylation reaction could be detected with maltose (G2) as substrate, but that maltotriose (G3), maltotetraose (G4) and maltopentaose (G5) were effective substrates, producing other maltooligosaccharides and glucose, but not maltose. Note that when maltopentaose was substrate, high molecular weight oligosaccharides were produced which remained at the origin during TLC. These results agree well with the results obtained by native potato enzyme (Figure 3.2) and with the reported action of D-enzyme on maltooligosaccharides, so demonstrating that recombinant D-enzyme has similar activity to the native enzyme.

4.3 Action of D-enzyme on high-molecular weight starch in the presence of acceptor molecule

Since the purified enzyme is free from hydrolytic activity, it is now possible to investigate the proposal that D-enzyme can transfer maltosyl groups from starch to glucose (Yoshio *et al.*, 1986; Lin and Preiss, 1988). Short chain amylose and amylopectin were incubated with enzyme for 4 h in the presence or absence of glucose (Figure 4.2). Oligosaccharides larger than maltotriose were produced from both substrates only in the presence of acceptor molecule, glucose. The results indicate that D-enzyme does use these high molecular weight starch molecules as donors and transfers maltooligosaccharide units to glucose. The amount of oligosaccharide product from amylopectin were less than from short chain amylose or maltooligosaccharides (Figure 4.1). It may indicate that amylopectin is not an effective donor molecule. However, it is also possible that non-reducing ends of

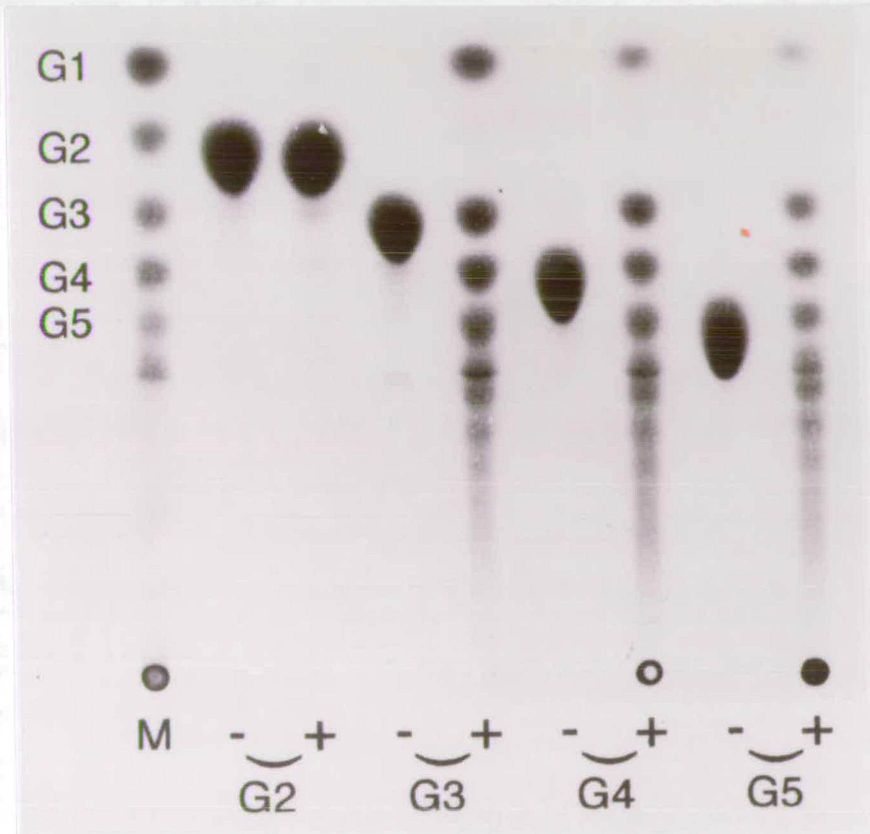


Figure 4.1 Thin layer chromatogram of reaction products from the activity of purified D-enzyme on various oligosaccharides.

The reaction mixture (30 µl) containing 300 µg of substrates was incubated with (+) or without (-) enzyme (0.1 units) at 37 °C for 18 h. 20 µl of the reaction mixture was analysed by thin layer chromatography and stained with H_2SO_4 in methanol. M, Standard maltooligosaccharides; G2, G3, G4 and G5 are maltose, maltotriose, maltotetraose and maltopentaose, respectively.

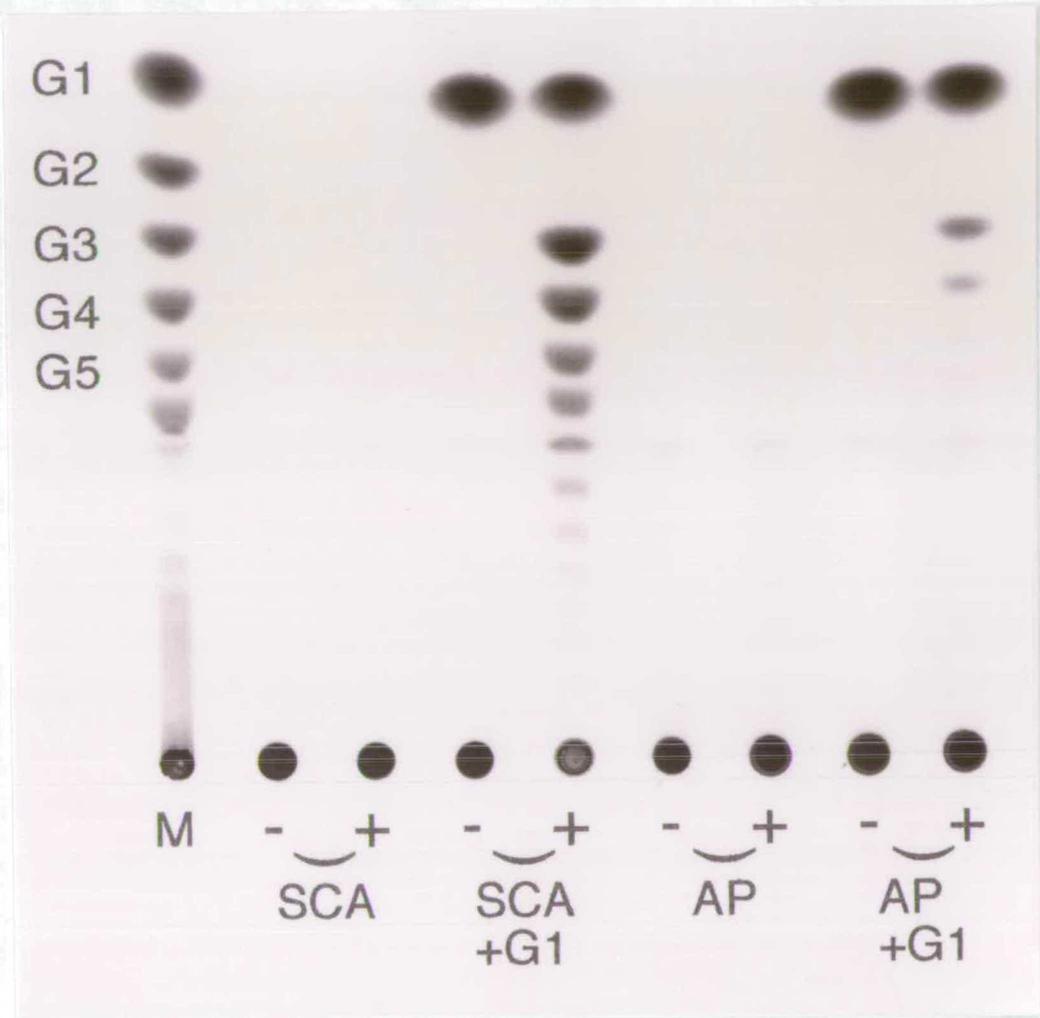


Figure 4.2 . Thin layer chromatogram of reaction products from the activity of purified D-enzyme on starch.

The reaction mixture (30 µl) containing 300 µg of substrates and the enzyme (0.01 units) was incubated with (+) or without (-) glucose (40 mM) at 37 °C for 4 h. 20 µl of the reaction mixture was analysed by thin layer chromatography and stained with H_2SO_4 in methanol. M, Standard maltooligosaccharides; G1, G2, G3, G4, G5, SCA and AP are glucose, maltose, maltotriose, maltotetraose, maltopentaose, short chain amylose (degree of polymerisation approximately 18, Amylose EX-I, Hayashibara Biochemical Laboratories, Japan) and amylopectin, respectively.

amylopectin side chains are better acceptor molecules than glucose, although an answer could not be obtained from this TLC analysis. The action of D-enzyme on high molecular weight starch in the absence of low molecular weight acceptor molecule is studied in the following section.

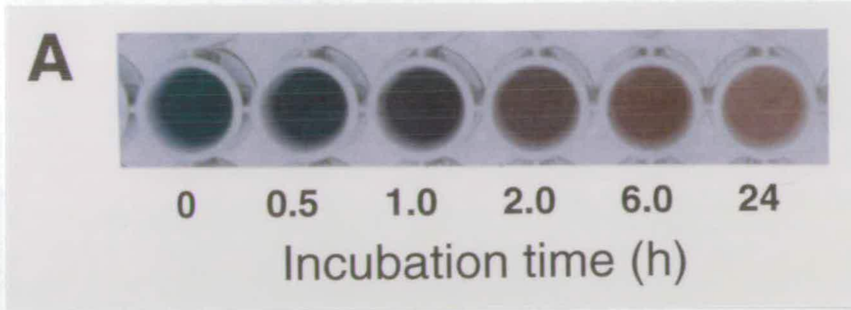
4.4 Action of D-enzyme on amylose

4.4.1 Effect of D-enzyme on synthetic amylose AS-320

Synthetic amylose AS-320 (average molecular weight of 320,000, and DP of 1975, Nakano Vinegar, Japan) was chosen to investigate the action of D-enzyme on high molecular weight amylose, since it is essentially free from α-1,6-linkages and has a narrow size distribution. Purified D-enzyme was incubated with synthetic amylose AS-320. During the reaction, the ability of amylose to form a blue complex with iodine (the blue value) decreased, and a brown product resulted instead (Figure 4.3).

This spectrum shift can not be easily understood from the known action of D-enzyme. Thus it may be caused by the action of D-enzyme itself, but may be caused by contaminating endogenous *E. coli* enzymes which cannot be detected by SDS-PAGE. In order to exclude the possibility of contaminating endogenous enzymes, enzyme samples were prepared from *E. coli* cells carrying plasmid pKK233-2 (without insert) or pKK233-DPE (with D-enzyme cDNA insert), and incubated with amylose AS-320. The spectrum shift of the amylose-iodine complex was only detected with the enzyme sample prepared from cells carrying pKK233-DPE. This result strongly suggested that this spectrum shift was caused by the D-enzyme gene product itself, and not by other contaminating endogenous *E. coli* enzymes.

In order to understand how this decrease of blue colour was caused by D-enzyme, the reducing power in each sample was measured and plotted with the blue value (A660 nm). As shown in Figure 4.4, A660 nm rapidly decreased during the initial few hours but the rate of decrease declined during further incubation. It is known that a similar spectrum shift of the amylose-iodine complex from blue to brown (or to yellow) is produced either by hydrolytic breakdown of amylose by amylases, or introduction of branched structure into amylose by branching enzyme. If this was caused by the hydrolytic breakdown of amylose, an increase of reducing power would be expected. However, no increase of reducing power was detected (Figure 4.4). If this spectrum shift was caused by branching enzyme, the number of



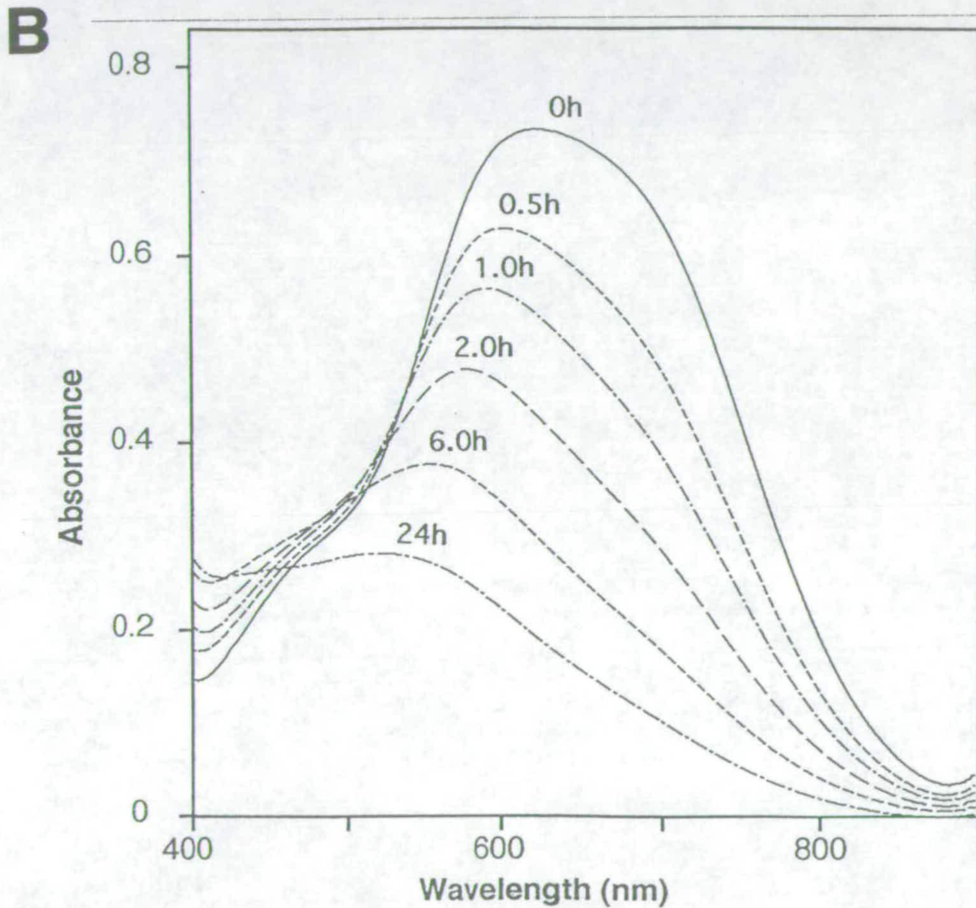


Figure 4.3 Effect of D-enzyme action on ability of amylose to form complex with Iodine
The reaction mixture (20 ml) containing 40 mg of synthetic amylose AS-320 (Nakano Vinegar Co., Ltd., Aichi, Japan) , 50 mM Sodium citrate buffer (pH 7.0), 100 mM of NaCl and 136 units of purified D-enzyme from recombinant *E. coli*, was incubated at 30 °C. At indicated time points 1 ml of the reaction mixture was removed and boiled for 5 min to stop the reaction then centrifuged. Fifty μl of the supernatant was mixed with 50 μl of distilled water and 2 ml of Iodine working solution, then photographed (A). The absorption spectrum was monitored using a Shimadzu UV-240 spectrophotometer (B).

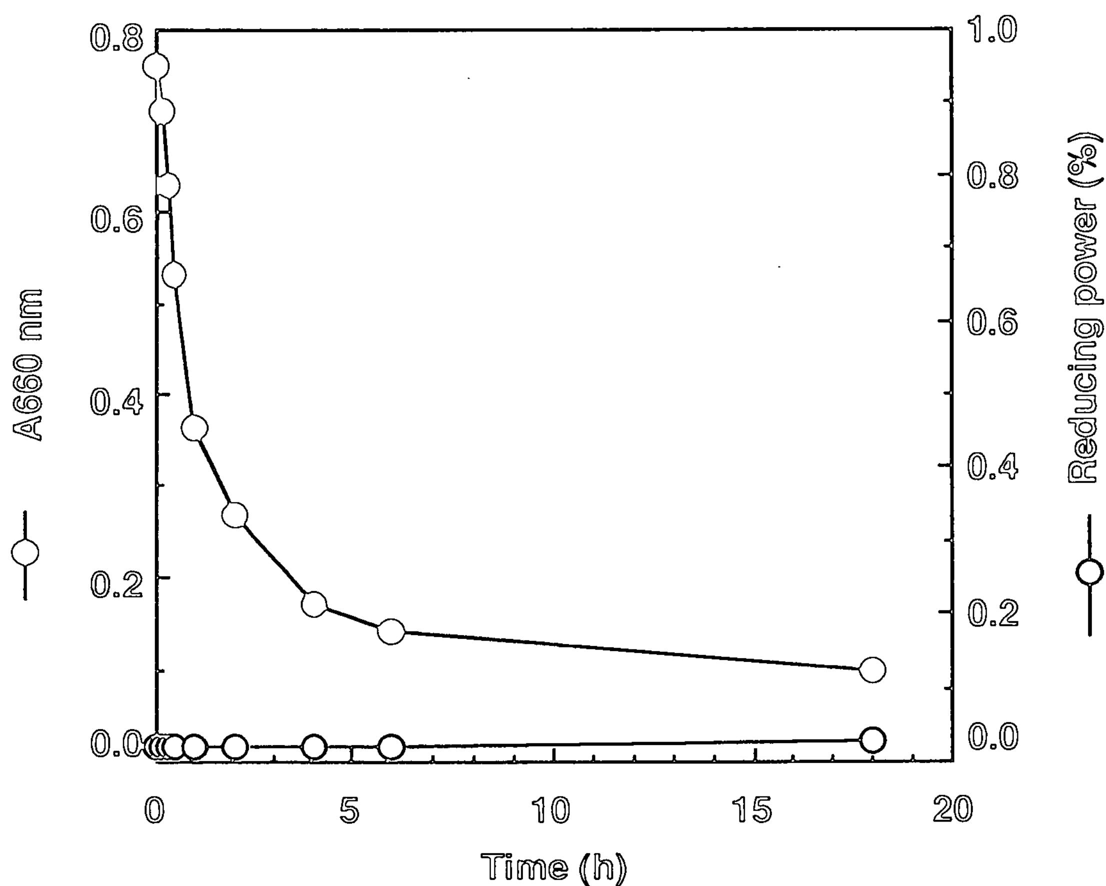


Figure 4.4 Absorption spectrum change of amylose-Iodine complex caused by D-enzyme
The reaction mixture (20 ml) containing 40 mg of synthetic amylose AS-320 (Nakano Vinegar Co., Ltd., Aichi, Japan) , 50 mM Sodium citrate buffer (pH 7.0), 100 mM of NaCl and 136 units of purified D-enzyme from recombinant *E. coli*, was incubated at 30 °C. At indicated time points 1 ml of the reaction mixture was removed and boiled for 5 min to stop the reaction then centrifuged. Fifty μl of the supernatant was mixed with 50 μl of distilled water and 2 ml of Iodine working solution to measure the absorbance at 660 nm, and 200 μl was used for reducing power measurement. Reducing power when all the amylose was broken down to glucose was defined as 100 %.

α-1,6-linkages should be increased. The number of α-1,6-linkages in the final products were quantitated by measuring the reducing ends after debranching enzyme treatment (Takeda *et al.*, 1993), but none was detected (not shown). These results suggested that the decrease of blue value was caused by an unknown activity of D-enzyme on the amylose molecule.

4.4.2 Effect of D-enzyme on the molecular mass of amylose

In order to investigate how this decrease in blue-value is caused by D-enzyme, structural changes of amylose were analysed by gel filtration chromatography. As shown in Figure 4.5, the narrow size distribution of amylose AS-320 was shifted to a broad distribution during the initial few minutes, but during prolonged incubation, products showed a narrow distribution in the low molecular weight region (average molecular weight of 15,000). Thus the decrease in the absorbance of the amylose-iodine complex was caused by the conversion of amylose into smaller molecular weight products. Since no increase of reducing power was detected (Figure 4.4), one possible explanation of this conversion is an intra-molecular transglycosylation reaction, or cyclisation reaction, of D-enzyme to produce cyclic α-1,4-glucans as shown in Figure 4.6.

4.4.3 Confirmation of the presence of glucoamylase resistant glucan in the products

The presence of cyclic α-1,4-glucan can be demonstrated by glucoamylase treatment. Glucoamylase is an exo-type amylase and hydrolyses both α-1,4 and α-1,6 linkages of starch to produce glucose from the non-reducing end of the substrate. Thus linear or branched glucans are completely broken down to glucose but glucans with cyclic structure, having no non-reducing end, should be resistant to the attack. The samples shown in Figure 4.5 were treated with glucoamylase and analysed with the same gel filtration columns (Figure 4.7B). The peak of intact amylose AS-320 (0 min) disappeared in response to the attack of glucoamylase, but glucoamylase resistant molecules were produced in the samples treated with D-enzyme. The amount of glucoamylase resistant molecules, and the average molecular weight are shown in Figure 4.8. The yield of glucoamylase resistant molecules increased from 0 to a maximum level (>90 %) in the initial 30 min, and then did not change. The average molecular weight of glucoamylase resistant molecule estimated by gel-filtration was initially about 70,000 but decreased with time, finally to about 15,000. The presence

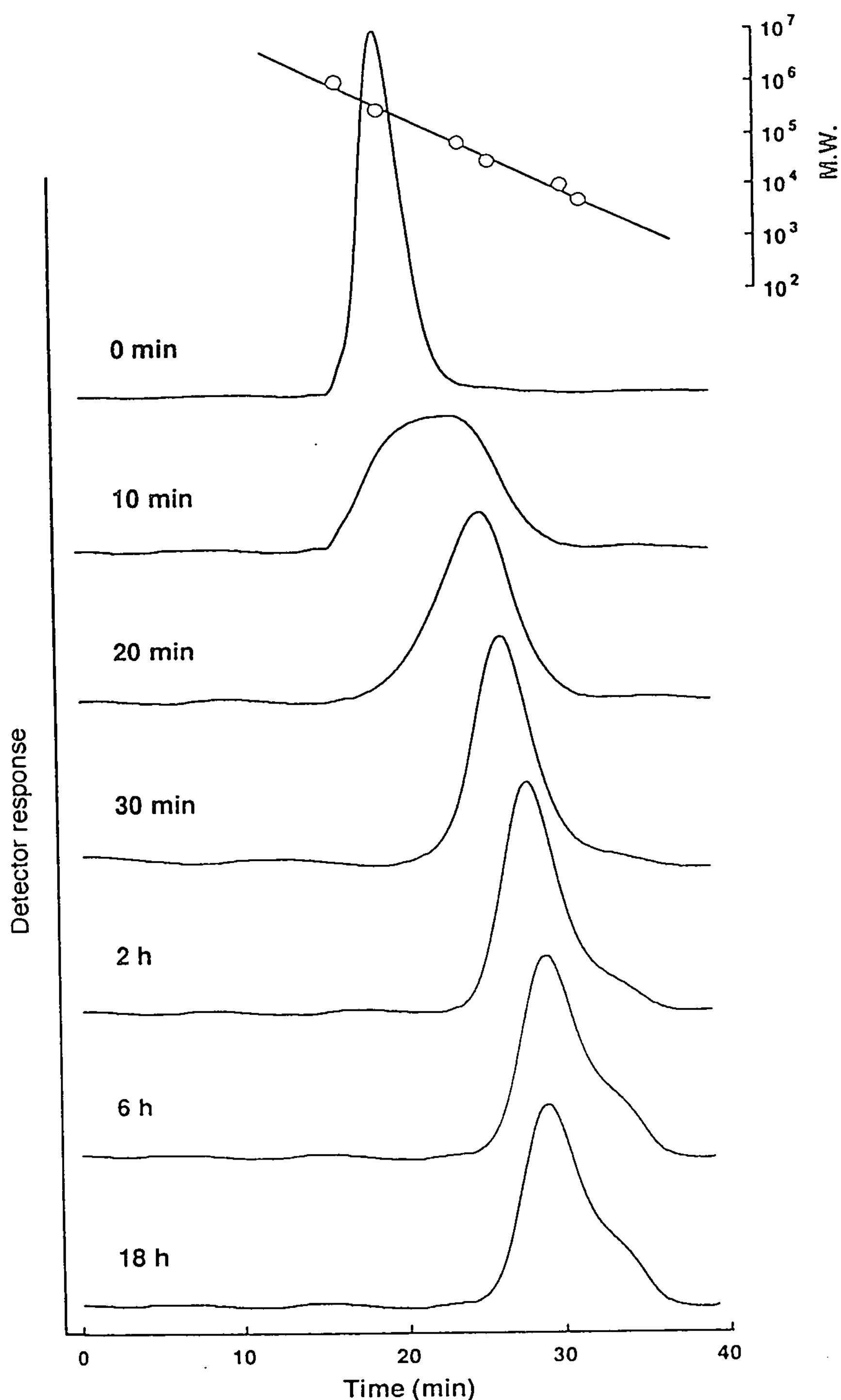


Figure 4.5 Gel-filtration chromatography of the products of D-enzyme action on amylose AS-320

Two hundred and fifty µl of the supernatant derived from Figure 4.4 was analysed by gel filtration chromatography using the Superose 6 prep grade column plus Superdex 30 column with flow rate of 1.0 ml min^{-1}. Standard curve for the molecular weight was produced using synthetic amylose with average molecular weights of 5, 10, 30, 70, 320 and 1000 kDa as standards.

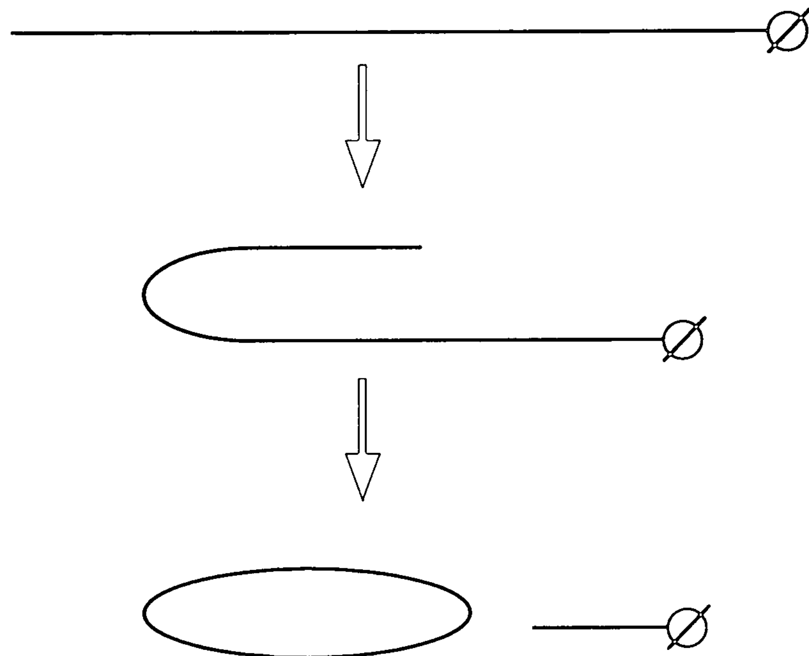


Figure 4.6 Diagrammatic representations of cyclisation reaction of D-enzyme on amylose

Linear or curved lines indicate α-1,4-glucan chains. Ø, Glucosyl residue at reducing end.

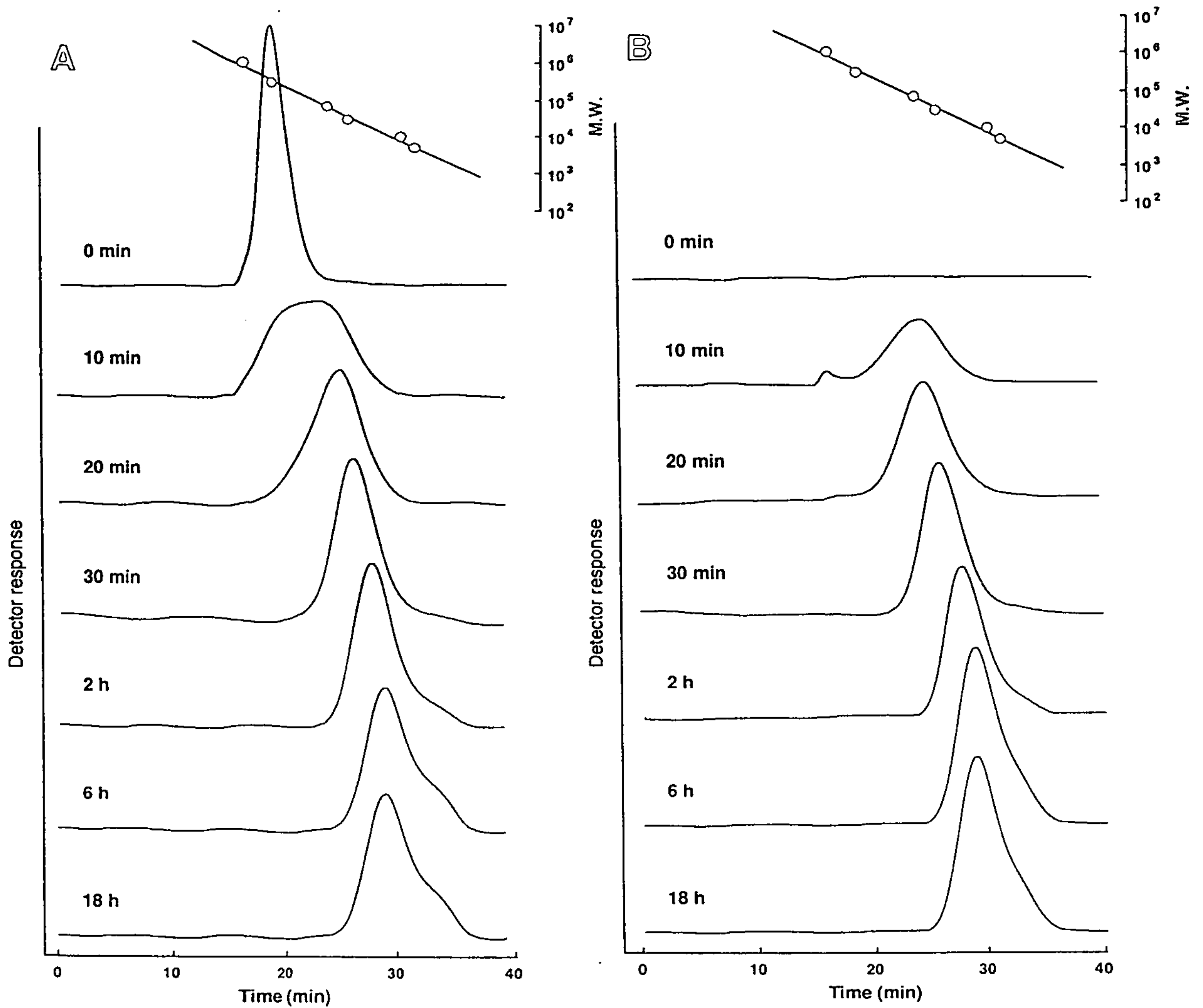


Figure 4.7 Gel-filtration chromatography of the products of D-enzyme action on amylose AS-320

Two hundred and fifty µl of the supernatant derived from Figure 4.4 was analysed before (A) and after (B) glucoamylase treatment, by gel filtration chromatography using the Superose 6 prep grade column plus Superdex 30 column with flow rate of 1.0 ml min^{-1}. Standard curve for the molecular weight was produced using synthetic amylose with average molecular weight of 5, 10, 30, 70, 320 and 1000 kDa as standards.

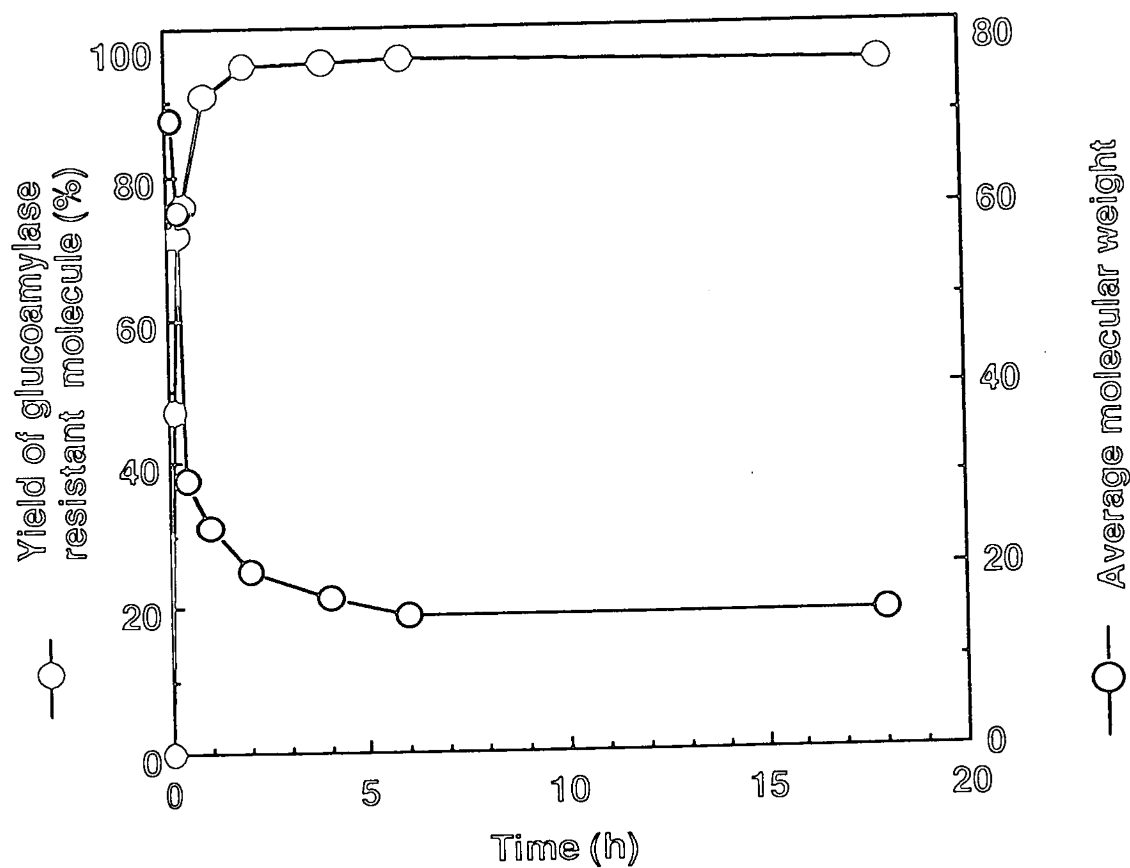


Figure 4.8 Yield and average molecular weights of glucoamylase resistant molecules produced by D-enzyme
Yield of glucoamylase resistant molecules was measured as described in section 2.4.7.3. Average molecular weight of glucoamylase resistant molecules was estimated by gel filtration chromatography using synthetic amylose with different average molecular weight as standards. (Figure 4.7).

of the glucoamylase resistant molecule strongly suggested that D-enzyme catalyses the cyclisation of amylose AS-320 to produce cyclic α-1,4-glucan.

4.4.4 Analysis of the structure of glucoamylase resistant molecules

It is well known that cyclodextrin glucanotransferase (CGTase) catalyses the cyclisation reaction of amylose or amylopectin to produce cyclomaltooligosaccharides with a degree of polymerisation (DP) of 6, 7 or 8, which are called α, β and γ-cyclodextrin, respectively. Thus, D-enzyme may produce cyclodextrins. However, the estimated average molecular weight of 15,000 for the glucoamylase resistant molecules indicates that they are not cyclodextrins but those with higher molecular weights.

Further structural analysis of the final products from amylose AS-320 was carried out by high performance anion exchange chromatography (HPAEC). The elution pattern of the products after 18 h D-enzyme treatment is shown in Figure 4.9A together with α-1,4-glucan standards (Figure 4.9D). The minor peaks eluted between 3 and 17 min had the same retention times as α-1,4-glucans with a DP of between 1 and 14, but the major peaks eluted after 17 min had different retention times from α-1,4-glucans. The nature of the products was first analysed by treatment with glucoamylase. The minor products of D-enzyme reaction eluted between 3 and 17 min were completely hydrolysed to glucose by glucoamylase (Figure 4.9B) . However, the major products with retention time greater than 17 min were resistant to glucoamylase. The products were next analysed by treatment with α–amylase, an endo-amylase which hydrolyses α-1,4-glucans to produce only glucose and maltose. If the glucoamylase resistance was introduced by anyway other than cyclisation, eg. branching or modification of the amylose molecule, additional degraded products other than glucose and maltose should be produced. The products of D-enzyme action were completely hydrolysed to glucose and maltose (Figure 4.9C), indicating that the products of D-enzyme reaction are all α-1,4-glucans without any modification or branching. It should also be noted that no peaks corresponding to cyclodextrins were detected in the glucoamylase resistant molecules (Figure 4.9C), indicating that putative cyclic α-1,4-glucan produced by D-enzyme are not cyclodextrins but have higher molecular weights.

The glucoamylase resistant products were then purified from glucose and size fractionated by gel filtration chromatography on a Superdex 30 column. The putative cyclic α-1,4-glucans in each fraction were precipitated with ten volumes of ethanol

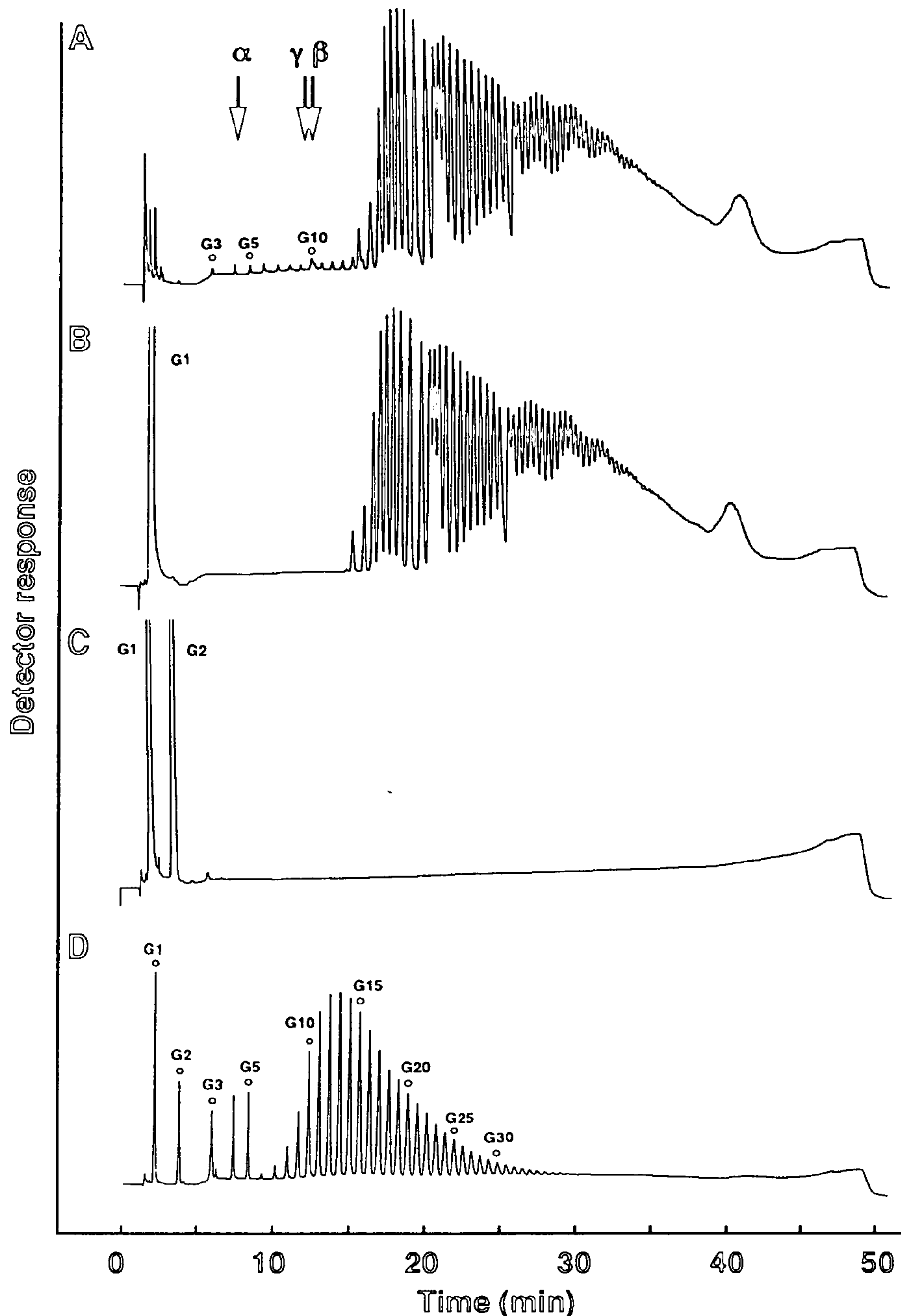


Figure 4.9 HPAEC analysis of the products of D-enzyme action on amylose AS-320

The products of D-enzyme treatment (18 h) on AS-320 were analysed with HPAEC using the condition described in section 2.4.5. Fifty μg of glucan either before any treatment (A), after glucoamylase treatment (B), after α–amylase treatment (C) was analysed. Short chain amylose was mixed with glucose and maltooligosaccharides, and used as α-1,4-glucan markers (D). Positions where α, β, γ-cyclodextrins were eluted are indicated by arrows. Numbers above and beside peaks (G1, G5 etc.) indicate the DP of products. The amylase treatments were carried out at pH 5.5 at 40 °C. The amount of pullulanase, isoamylase, glucoamylase and α-amylase used was 1.0, 2.0, 50 and 10 units / μg-glucan, respectively.

then lyophilised. Quantitation of reducing or non-reducing ends of the glucoamylase resistant molecules in each fraction were carried out using the rapid Smith degradation method or Park-Johnson method, respectively, but none was detected (not shown), strongly indicating a cyclic structure.

Time of flight mass spectrometry (TOF-MS) was next employed to demonstrate the cyclic structure as well as to determine the DP of the smallest glucoamylase resistant molecule. TOF-MS is very useful since molecular mass of not only single molecules but also those in a mixture of several molecules can be obtained. A glucan with DP of n in any non-cyclic structure should have the molecular mass of 162x(n)+18, whereas a glucan with DP of n in any cyclic structure should have a mass of 162x(n). The elution pattern on HPAEC and the TOF-MS spectrum of the glucoamylase resistant molecules in the smallest fraction of Superdex 30 chromatography is shown in Figure 4.10. Several peaks were obtained in TOF-MS spectrum and the molecular mass agreed with the theoretical value for cyclic G17, G18, G19, and longer. From this result and the results before, it is concluded that the smallest glucoamylase resistant molecule (peak A) is the cyclic α-1,4-glucan with DP of 17, and the consecutive peaks (B, C, D and so on) are cyclic glucans with DP of one unit longer for each successive peak.

This hypothesis was finally confirmed by conversion of cyclic molecules to linear molecules by partial acid hydrolysis. Peaks G to J of glucoamylase resistant products shown in Figure 4.10A were purified by HPAEC then partially hydrolysed with 0.1 N HCl. Products from peak G hydrolysis eluted as linear molecules with a DP from 1 to 23 (Figure 4.11A). The largest product with a DP of 23 (G23) is assumed to have resulted from hydrolysis of one glucosidic linkage of the peak G molecule. Similar results were obtained for all other peaks except that the DP of the longest linear product was one unit longer for each successive peak (Figure 4.11B-D). The retention time of each peak was less than that of the largest linear product. Therefore these results show that D-enzyme catalyses the intra-molecular transglycosylation reaction on amylose, as schematically shown in Figure 4.6, to produce cyclic α-1,4-glucans (cycloamylose) with a DP ranging from 17 (Figure 4.10A, peak A) to a few hundred (Figure 4.7).

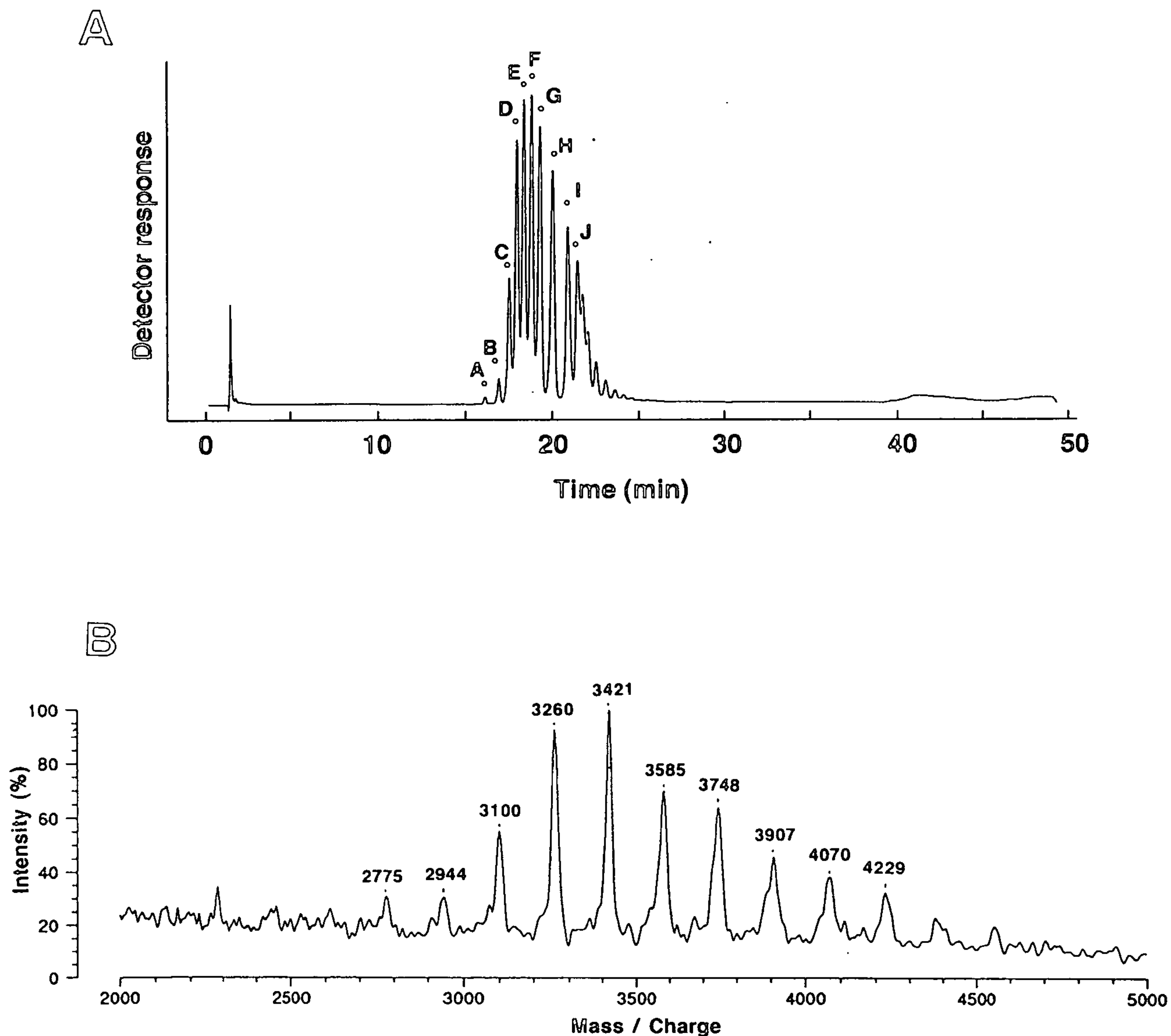


Figure 4.10 TOF-MS analysis of the glucoamylase resistant molecules Glucoamylase resistant molecules were size fractionated by gel filtration chromatography as described in section 2.4.7.4, and the fraction containing the smallest glucans was used for TOF-MS analysis. A, HPAEC analysis of the glucoamylase resistant molecules used for TOF-MS analysis. The glucoamylase-resistant molecules were labelled A to J as shown. B, TOF-MS spectrum. Numbers on each peak indicate the molecular mass of the glucan + 23 (Na).

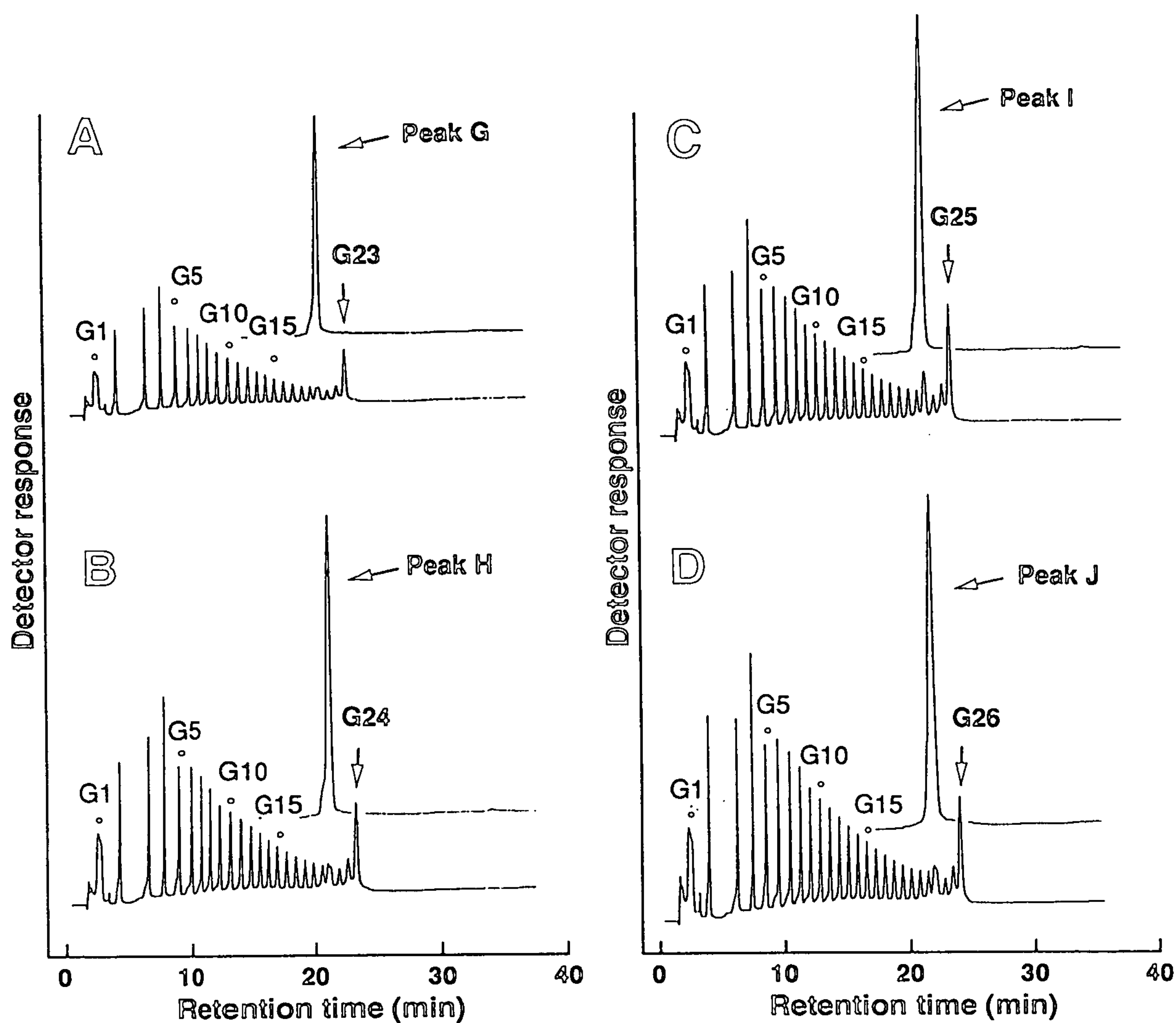


Figure 4.11 Partial acid hydrolysis of glucoamylase-resistant molecules
Glucoamylase resistant molecules of peaks G to J in Figure 4.10A were purified by the HPAEC system as described in section 2.4.7.5. The elution profiles of each purified peak, before (upper line) and after (lower line) partial acid hydrolysis are shown. Numbers above peaks (G1, G5 etc.) indicate the DP of products, with the largest product indicated by an arrow.

4.4.5 Action of D-enzyme on cyclic α-1,4-glucan

Figures 4.7 and 4.8 indicated that large cyclic α-1,4-glucans which were initially produced by D-enzyme were converted into smaller cyclic α-1,4-glucans by prolonged D-enzyme action. The fact that reducing power did not increase during the reaction (Figure 4.4), suggested this conversion was not caused by hydrolytic action but occurred by transglycosidic action of D-enzyme as illustrated in Figure 4.12. One α-1,4-glucosidic linkage within the large cyclic α-1,4-glucan produced in the early stage of the enzyme action could be cleaved by D-enzyme then transferred to the linear donor molecule to produce long linear amylose. This long linear amylose is now attacked again by D-enzyme and the smaller cyclic α-1,4-glucan produced through the cyclisation reaction (Figure 4.6). The resultant linear amylose can be attacked again by D-enzyme to produce another small cyclic α-1,4-glucan. This model was confirmed by the following experiment. Large cyclic α-1,4-glucan (average molecular weight of 30,000) was prepared by gel filtration on a superdex 30 column as described in section 4.4.5. This large cyclic α-1,4-glucan was incubated with D-enzyme in the absence or in the presence of acceptor molecule, glucose. According to the model, smaller products should be produced only in the presence of acceptor molecule, but none should occur in the absence of acceptor molecule. As shown in Figure 4.13, no smaller products were produced in the absence of glucose (Figure 4.13A), but smaller products were produced in the presence of glucose (Figure 4.13B-E). When a large amount of glucose was added, smaller molecules were produced (Figure 4.13D,E). When a small amount of glucose was added, larger molecules including cyclic α-1,4-glucans were produced (Figure 4.13B,C). The presence of smaller cyclic α-1,4-glucans within these degraded molecules was confirmed by analysing the glucoamylase resistant molecules by the same gel-filtration column (Figure 4.13G,H,I). These results suggested that D-enzyme catalyses the transglycosidic liniarisation of cyclic α-1,4-glucan as shown in Figure 4.12.

4.4.6 Proposed action of D-enzyme on amylose

The results described above clearly demonstrate novel activities of D-enzyme. Firstly, it can catalyse an intra-molecular transglycosylation reaction on high molecular weight amylose to produce cyclic α-1,4-glucans with a DP ranging from 17 to several hundred. Secondly, high molecular weight amylose can serve as donor and acceptor. Thirdly, very long α-1,4-glucan units can be transferred by the enzyme. Fourthly, it catalyses the transglycosidic linearisation of cycloamylose when acceptor is present.

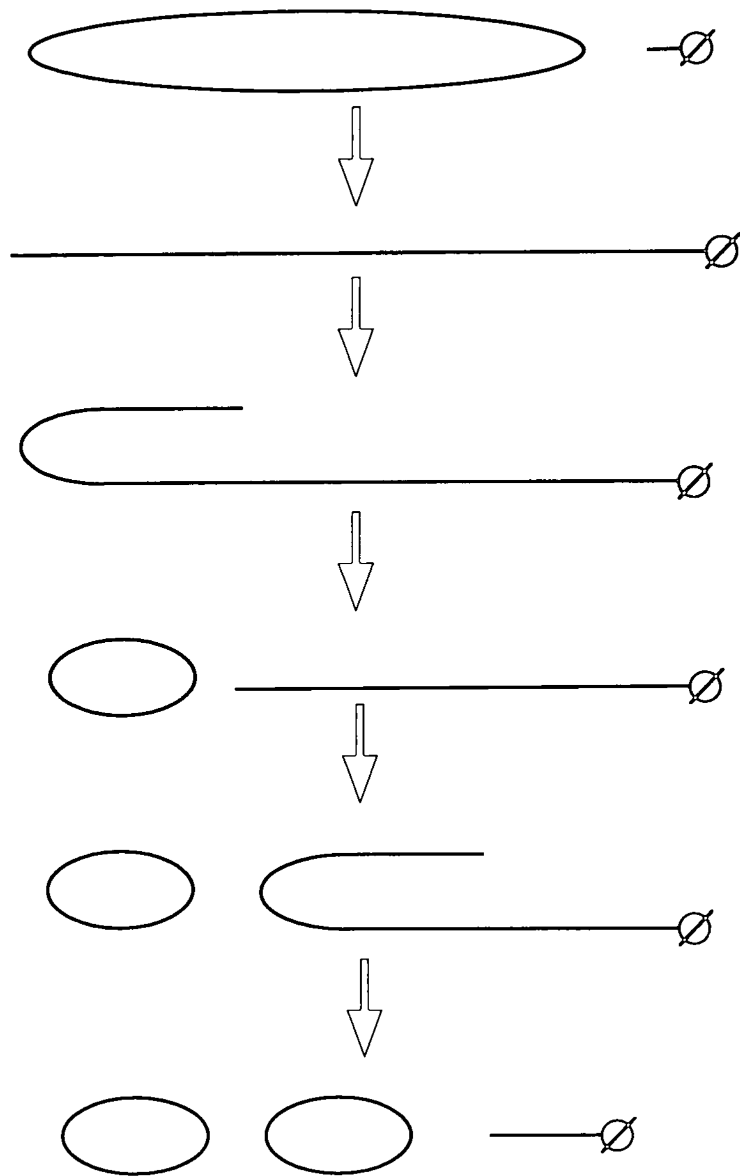


Figure 4.12 Diagrammatic representations of action of D-enzyme on cyclic α-1,4-glucan in the presence of acceptor molecule
Lines and circles indicate α-1,4-glucan chains, where the relative length represents their relative DP. ∅, Glucosyl residue at reducing end.

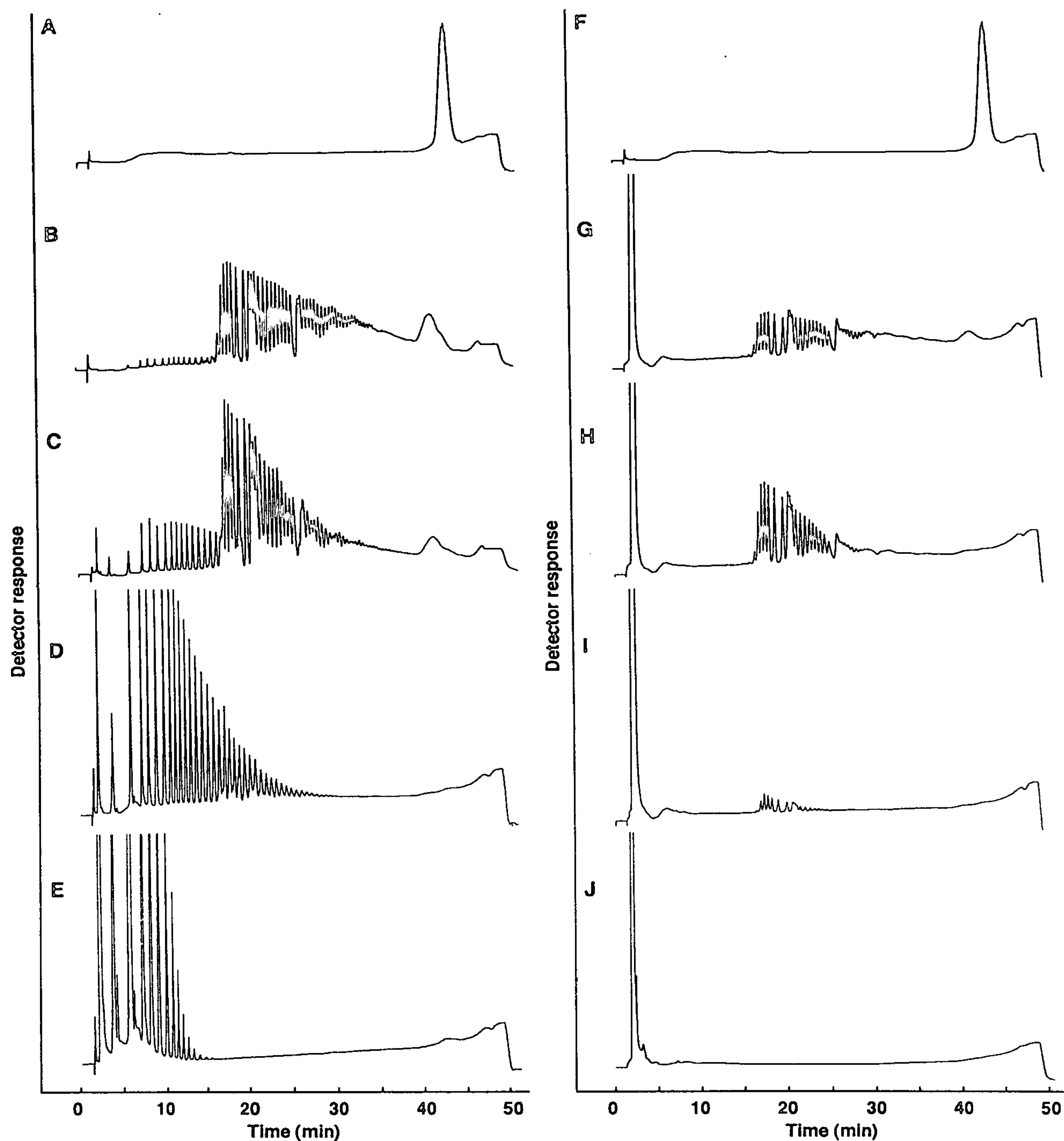


Figure 4.13 Analysis of the action of D-enzyme on cyclic α-1,4-glucan in the presence and absence of acceptor molecule

Cyclic α-1,4-glucan with average molecular weight of 30 kDa was treated with D-enzyme in the absence (A, F) of glucose, or in the presence of glucose at the weight ratio (glucose / cyclic glucan) of 0.0005 (B, G), 0.005 (C, H), 0.05 (D, I) and 0.5 (E, J). The reaction mixture were incubated at 30 °C for 3 h then boiled to terminate the reaction. The products were analysed by HPAEC before (A, B, C, D and E) and after (F, G, H, I and J) glucoamylase treatment.

These activities of D-enzyme are explained in Figure 4.14. The reaction starts with D-enzyme attacking an α-1,4-linkage. The enzyme then transfers the newly formed reducing end of the substrate either to the non-reducing end of a separate linear acceptor molecule or glucose (the intermolecular transglycosylation or disproportionation reaction), or to its own non-reducing end (the intramolecular transglycosylation or cyclisation reaction). The reversibility of these reactions allows large cyclic molecules to be linearised again by transglycosylation, and smaller cyclic molecules to be produced subsequently (Figure 4.14). Apparently the equilibrium tends towards the formation of cycloamylose with a M_r of about 15,000 (DP of 90), as shown in Figure 4.7.

4.5 Action of D-enzyme on amylopectin

4.5.1 Effect of D-enzyme on the molecular mass of amylopectin

Waxy corn starch was chosen to study the effect of D-enzyme on amylopectin. The starch was dissolved and incubated with recombinant potato D-enzyme purified from *E. coli* MC1061Δ*glg*, for up to 40 h and fractionated by gel filtration chromatography. The peak of intact amylopectin eluted with the exclusion volume (Figure 4.15). Upon incubation with D-enzyme, products of lower molecular mass were detected, and after 40 h the products were represented by two peaks, fractions I and II (average M_r 30,000 and 3,000 respectively). Fraction II appeared earlier in the incubation than fraction I (Figure 4.15). Since purified D-enzyme does not contain any hydrolytic activity, it was considered possible that the production of low molecular weight molecules from amylopectin was due to the cyclisation activity of D-enzyme.

In order to test for the presence of cyclic products, the resistance against glucoamylase treatment was investigated. As shown in Table 4.1, amylopectin was almost completely (98.6 %) degraded to glucose by the glucoamylase, but after 40 h incubation with D-enzyme, only 68.3 % was glucoamylase sensitive. Most of the glucoamylase-resistant products were found in fraction II (Table 4.1). These observations suggested that cyclic molecules had been produced. The combination of glucoamylase and debranching enzyme released more glucose, indicating that some glucoamylase-resistant products contained α-1,6 links (Table 4.1).

4.5.2 Quantitative analysis of products from amylopectin

The possible products of intramolecular transglycosylation reactions which D-enzyme might catalyse, based on our knowledge of its action on amylose and the

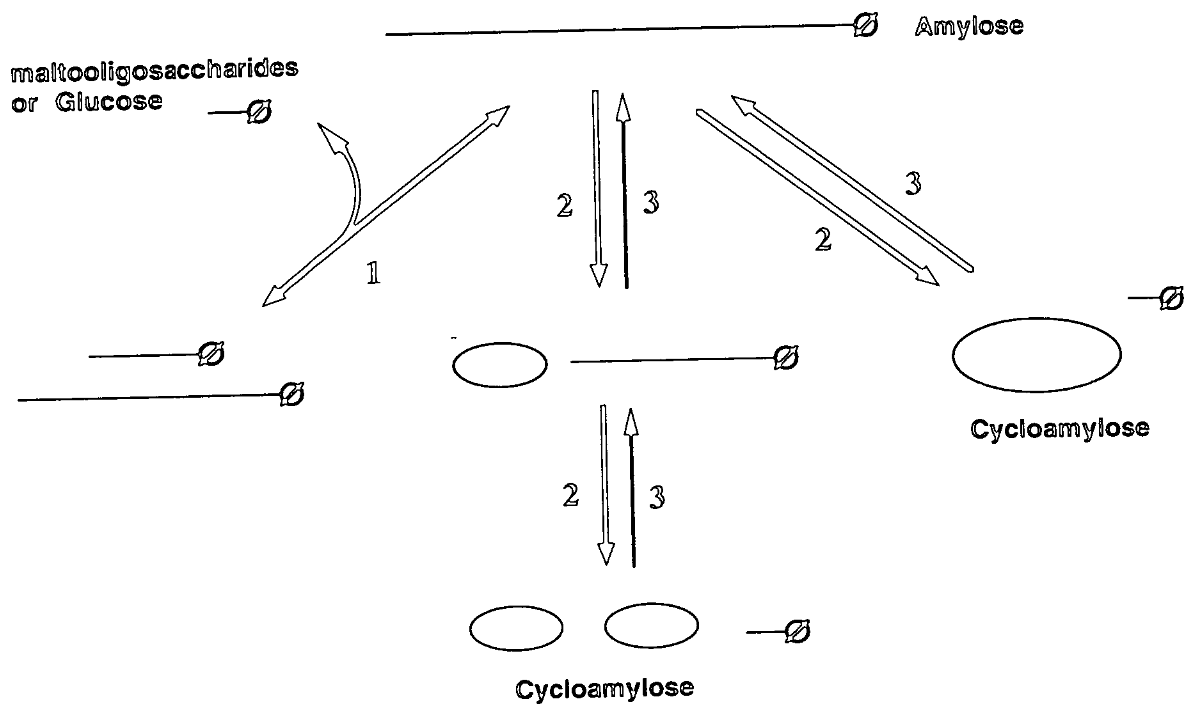


Figure 4.14 Diagrammatic representations of action of D-enzyme on amylose and cyclic amylose

Thin lines and circles indicate α-1,4-glucan chains, where the relative length represents their relative DP. Ø, Glucosyl residue at reducing end. Reaction 1 is the disproportionation reaction (intermolecular transglycosylation). Reaction 2 is the cyclisation reaction (intramolecular transglycosylation). Reaction 3 is the transglycosidic linearisation reaction (intermolecular transglycosylation).

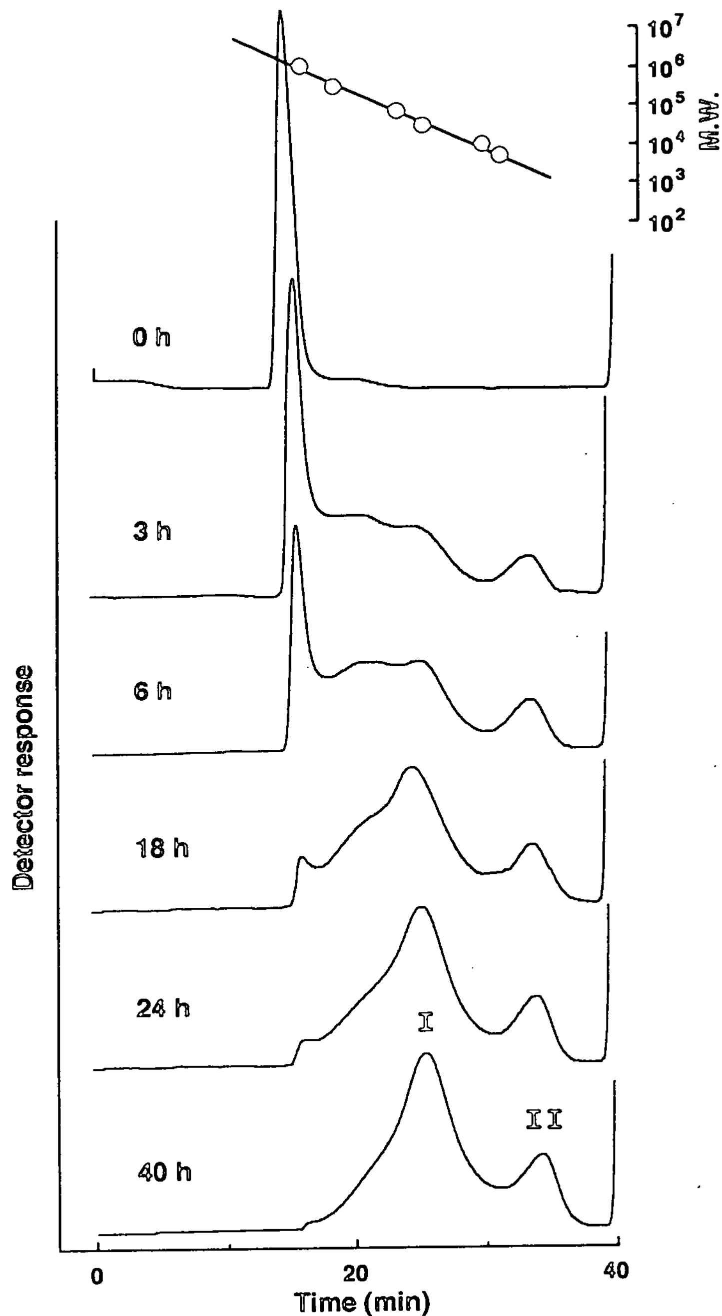


Figure 4.15 Gel-filtration chromatography of the products of D-enzyme action on amylopectin

The reaction mixture (10 ml) containing 20 mg of waxy corn starch (Nihon Shokuhin Kako Co., Ltd., Tokyo, Japan) , 10 mM Sodium citrate buffer (pH 7.0), 9 % (v/v) of dimethyl sulfoxide and 340 units of purified D-enzyme from recombinant *E. coli*, was incubated at 30 °C. At indicated time points 600 μl of the reaction mixture was removed and boiled for 5 min to terminate the reaction then centrifuged. Supernatant (250 μl) was analysed by gel filtration chromatography using the Superose 6 prep grade column plus Superdex 30 column with flow rate of 1.0 ml min^{-1}. Standard curve for the molecular weight was produced using synthetic amylose with average molecular weights of 5, 10, 30, 70, 320 and 1000 kDa as standards. Two peaks obtained in the 40 h sample were labelled peak I and II as shown. Strong detector response around 40 min was derived from dimethyl sulfoxide in the sample.

Table. 4.1 Yield of glucose produced by glucoamylase treatment on the products from amylopectin by D-enzyme action

	0 (h)	40 (h)		
		Total	Peak I	Peak II
Yield of glucose (%)				
Glucoamylase	98.6	68.3	84.5	20.0
Glucoamylase + debranching enzymes	98.8	84.8	97.4	36.9
Glucoamylase + α-amylase	100	100	100	100

Table. 4.2 Yield of products from the action of D-enzyme on amylopectin

	0 (h)	40 (h)		
		Total	Peak I	Peak II
Yield of products (%)				
Non cyclic part of glucan	98.6	68.3	84.5	20.0
Cyclic glucan (α-1,4- only)	1.2	15.2	2.6	63.1
Cyclic glucan (α-1,4- and α-1,6-)	0.2	16.5	12.8	16.8

results in Table 4.1 are summarised in Figure 4.16. It also shows how glucoamylase, debranching enzyme and α-amylase can be used to quantitate the amount of each product. The results of such analyses are shown in Table 4.2 and indicate that D-enzyme catalyses the formation of cyclic glucans with α-1,4 links only (mainly in fraction II), and cyclic glucans containing both α-1,4 links and α-1,6 links (mainly in fraction I).

4.5.3 Structural analysis of products from amylopectin

Glucans in each fraction were further analysed by HPAEC (Figure 4.17). Fraction I products were not resolved by HPAEC (Figure 4.17A) but after treatment with debranching enzyme, molecules with retention times equivalent to linear glucans were observed (Figure 4.17B). These linear glucans were shorter than those of the original amylopectin substrate (Figure 4.17F). The glucoamylase-resistant molecules from fraction I had retention times similar to cyclic α-1,4 glucans but did not resolve clearly, indicating that other structures may be present (Figure 4.17C). Since most of these glucoamylase-resistant molecules became glucoamylase-sensitive when treated with debranching enzyme (Figure 4.17D), they are apparently mostly cyclic glucans containing α-1,6 links. This conclusion is supported by the observation that treatment of the glucoamylase-resistant molecules with α-amylase, produced not only glucose and maltose, but other oligosaccharides (Figure 4.17E).

Fraction II contained molecules which eluted with retention times similar to α-1,4 glucans (Figure 4.17G). Treatment with debranching enzyme released some glucose, maltose and maltooligosaccharides (Figure 4.17H) indicating the presence of very short α-1,6-linked branches. The glucoamylase-resistant fraction eluted as linear α-1,4 glucans with DPs from 14 up to at least 40 (Figure 4.17I) and most of them were still glucoamylase-resistant after treatment with debranching enzyme (Figure 4.17J) showing them to be cyclic α-1,4-glucans without α-1,6-links. Furthermore, the glucoamylase-resistant molecules were degraded largely to glucose and maltose by α-amylase (Figure 4.17E). These observations all indicate that fraction II contains mainly cyclic α-1,4-glucans without α-1,6-links, but also some cyclic glucans with α-1,4 and α-1,6 links.

4.5.4 Proposed action of D-enzyme on amylopectin

From the results above, a model for the *in vitro* action of D-enzyme on amylopectin is proposed (Figure 4.18). Fraction II appears before fraction I, and contains mainly (63 %) cyclic α-1,4-glucan. We propose that molecules in fraction II

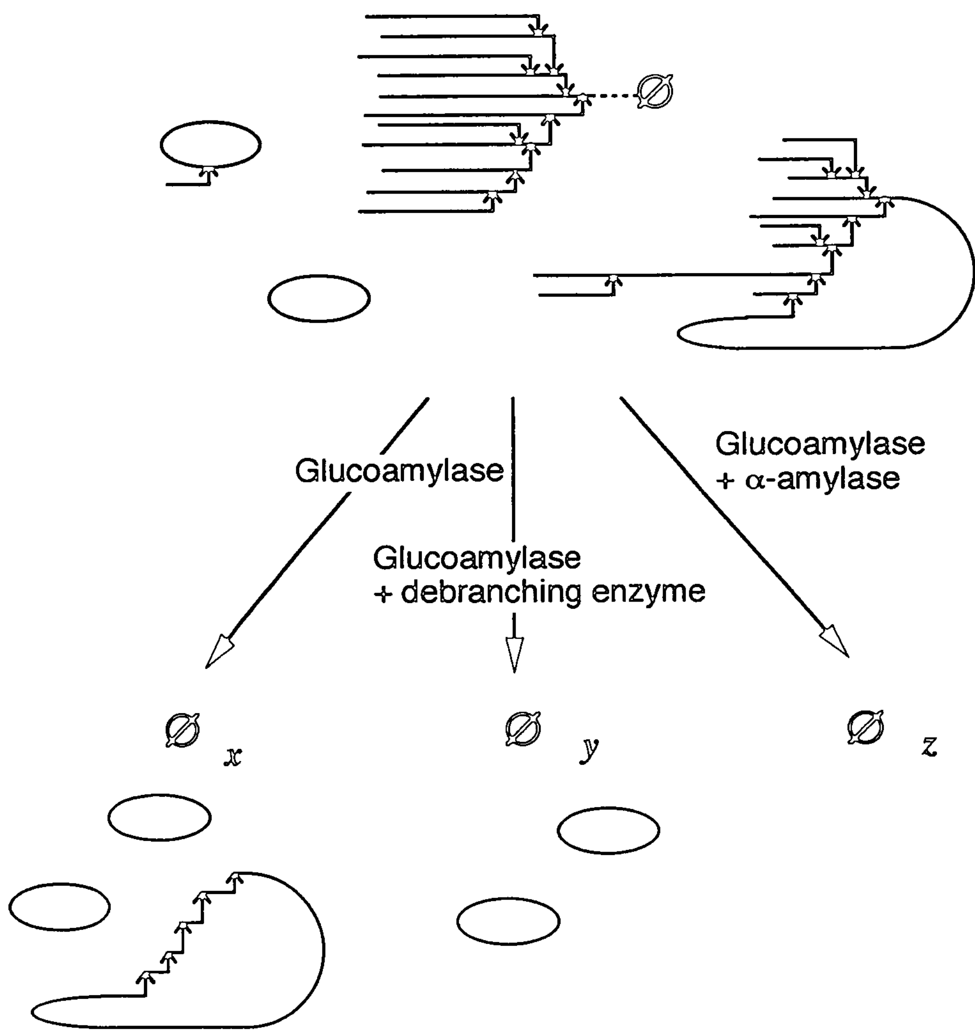


Figure 4.16 Schematic illustration of the quantitation of branched and unbranched cyclic glucans

The amount of glucan not involved in cyclic structure, glucan involved in α-1,4-linked cyclic structure, and glucan involved in α-1,4-, and α-1,6-linked cyclic structure can be determined by measuring the amount of glucose produced from the glucan by the different combinations of amylases as described in section 2.4.8.2. Linear or curved lines indicate α-1,4-glucan chains. Vertical arrows indicate α-1,6-branch points. Ø, Glucosyl residue at reducing end. x, amount of glucose produced by glucoamylase treatment; y, amount of glucose produced by glucoamylase and debranching enzyme treatment; z, amount of glucose produced by glucoamylase and α–amylase treatment.

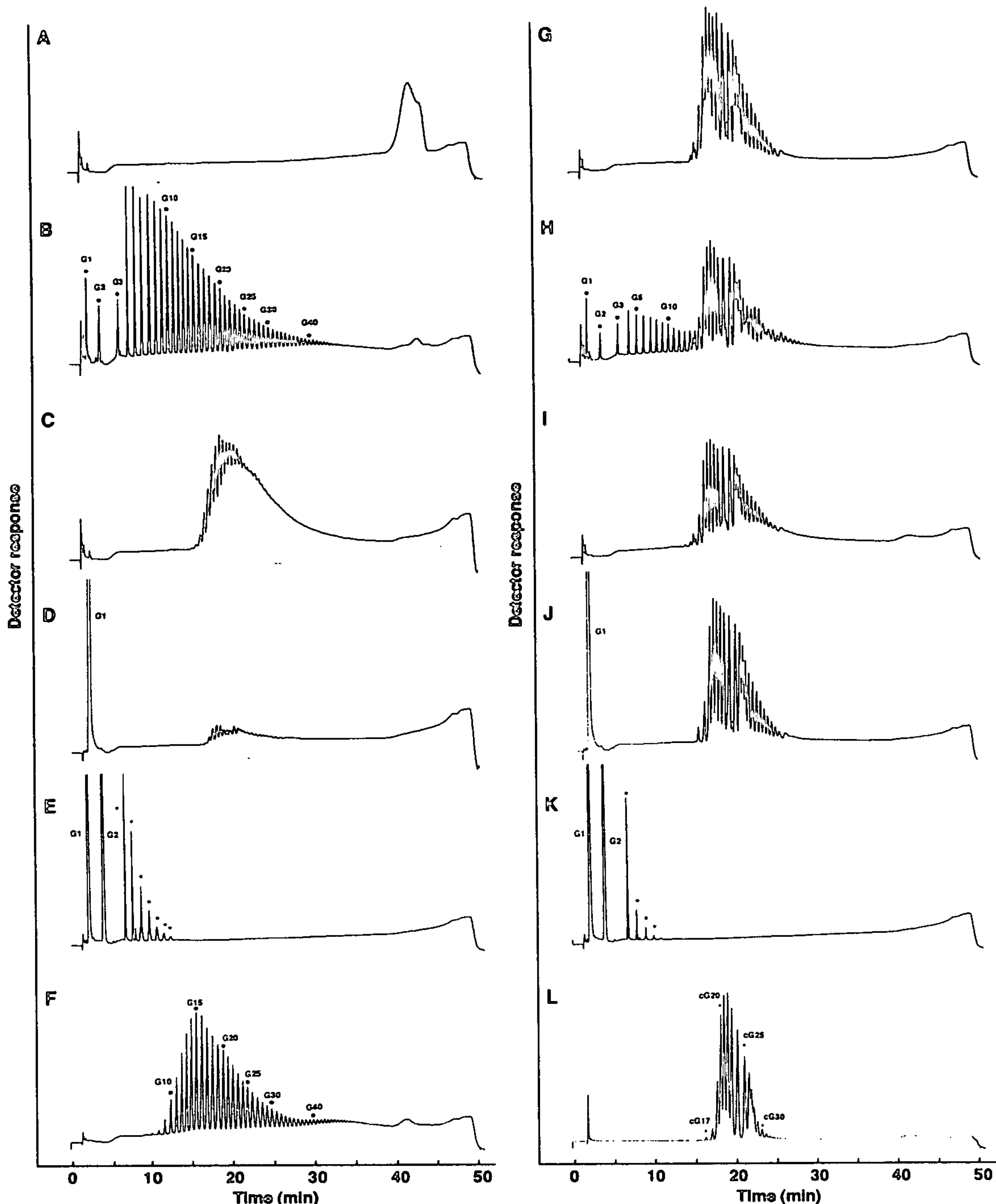
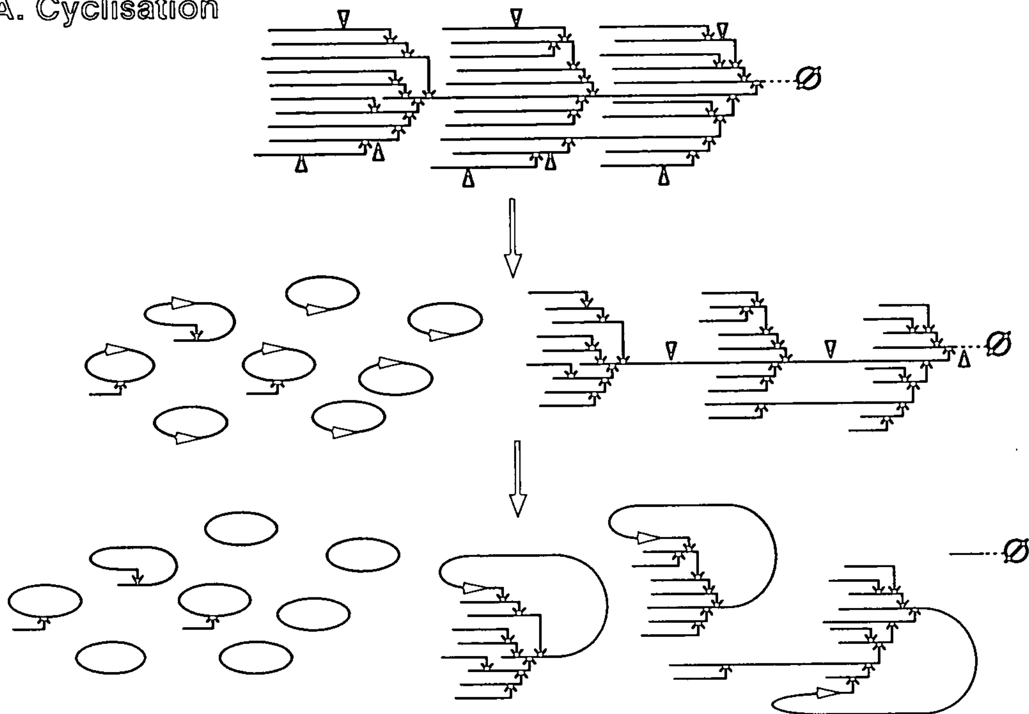


Figure 4.17 HPAEC analysis of the two major components produced by D-enzyme from amylopectin
Two major components (peak I and II, Figure 4.15) produced by D-enzyme from amylopectin were sub fractionated by gel-filtration. Forty μg of each component before (A, G) and after (B, H) debranching treatment were analysed by HPAEC. Glucoamylase resistant glucan was purified from each component as described in section 2.4.8.2, and 40 μg of each was analysed without any treatment (C, I), after glucoamylase and debranching enzyme treatment (D, J) or after α–amylase treatment (E, K). Waxy corn starch treated with debranching enzymes (F) and cycloamylose with DP of 17 to 32 (L) are shown as standards. A-E, the analysis for peak I, G-K, the analysis for peak II. Numbers above and beside peaks (G1, G5 etc.) indicate the DP of products.



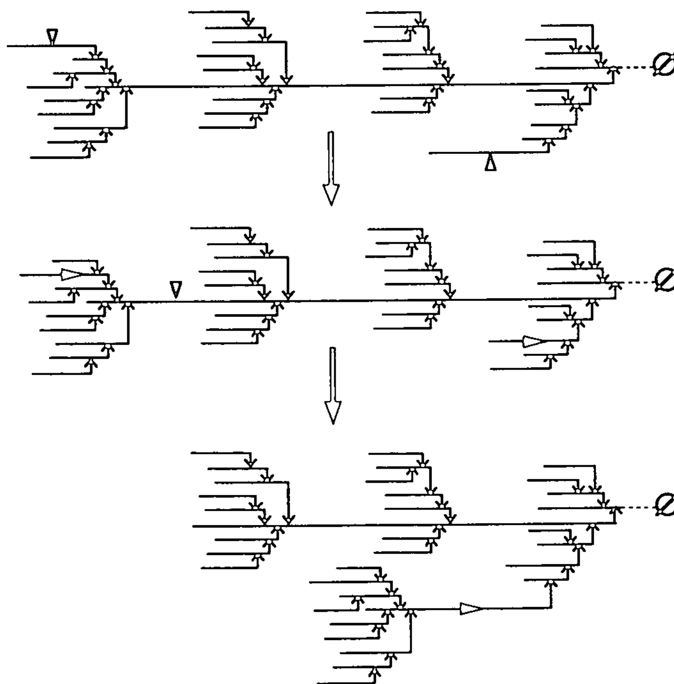


Figure 4.18 Proposed action of D-enzyme on amylopectin
Linear or curved lines indicate α-1,4-glucan chains. Vertical arrows indicate α-1,6-branch points. Open and solid triangles indicate the α-1,4-linkages attacked and newly synthesised by D-enzyme. Ø Glucosyl residue at reducing end.

were derived from the outer chains of the amylopectin molecule by transglycosylation, as shown (Figure 4.18A). Subsequently transglycosylation reactions involving inner chains would separate, from the high molecular weight amylopectin molecule, cluster units which contain short side chains but no non-reducing end (Figure 4.18A).

These results also suggest the following novel activity of D-enzyme. First, it catalyses the intra-molecular transglycosylation (cyclisation reaction) on amylopectin to produce cyclic α-1,4-glucan or branched glucan with cyclic structure. Second, both outer chains of the amylopectin molecule and inner chains of amylopectin can serve as donor. Third, highly branched glucan chain can be transferred by the enzyme. Furthermore, it may be speculated that D-enzyme may catalyse the disproportionation of amylopectin by transferring a α-1,4-glucan unit from one outer chain to another, or cluster unit from one place to another site (Figure 4.18B), although these activities are not demonstrated in the experiments above.

4.6 Discussion

4.6.1 Comparison of CGTase and D-enzyme

Results obtained in this chapter indicated that D-enzyme catalyse the cyclisation and disproportionating reaction on α-1,4-glucan, and transglycosidic linearisation of cyclic α-1,4-glucan in the presence of acceptor molecule, glucose. CGTase has been known to catalyse cyclisation and disproportionating reactions on α-1,4-glucans (French, 1957; Schmid, 1989). The transglycosidic linearisation of cyclodextrin in the presence of a suitable acceptor has also been demonstrated (the 'coupling reaction') (French, 1957). In all these respects, D-enzyme and CGTase seem to catalyse the same reaction, but the major difference is the DP of the cyclic α-1,4-glucans produced. CGTase produces cyclic maltodextrin (cyclodextrins) with DPs of 6, 7 or 8. Larger cyclodextrins with DPs of 9 to 13 have been reported to be produced, but only in trace amounts (Pulley and French, 1961; French *et al.*, 1965). On the other hand, D-enzyme produces cyclic α-1,4-glucans with DPs from 17 to several hundred, and never produces cyclodextrins. In spite of this similarity found in their activity, D-enzyme and CGTase shows very low similarity in their primary sequences as described in section 3.6.2. Thus, CGTase and D-enzyme may have distinct active site structures such that the cyclodextrin molecule fits that of CGTase but not that of D-enzyme, and cyclic α-1,4-glucan with DP larger than 17 fits only the D-enzyme active site. The tertiary structure of CGTase has already been obtained by X-ray crystallographic studies (Klein and Schulz, 1991; Kubota *et al.*, 1994; Lawson *et al.*,

1994; Knegtel *et al.*, 1996), so it would be very interesting to know the structure of D-enzyme, so that we may understand how the DP of cyclisation products are differently controlled by these two enzymes.

4.6.2 Novel cyclic glucan produced by D-enzyme

Starch is considered to be comprised of two components: amylose, a linear α-1,4-glucan, and amylopectin, an α-1,4-glucan containing α-1,6-linked branches. We now find that we should consider the possibility that cyclic glucans discovered in this chapter, cyclic α-1,4-glucan and branched glucan with cyclic structure, constitute other components of starch. At present it is not known if cyclic glucans are present in plant starch-accumulating tissues, such as seeds, fruits, tubers or leaves. However, D-enzyme is known to be present in various plant tissues and to be located inside the plastid where starch is accumulated.

The non-reducing property of cyclic glucans might be important for plant tissues, as is the case for non-reducing sugars such as sucrose, sorbitol and sugar phosphates. Furthermore, cyclic α-1,4-glucan is highly soluble in cold water (unlike linear amylose which is insoluble), which may also be an important property for starch metabolism *in vivo* .

4.6.3 Novel activity of D-enzyme on high molecular weight starch and the possible roles of D-enzyme in starch metabolism

Small oligosaccharides have been mainly employed as substrates for the analysis of D-enzyme activity *in vitro* and have been believed to be the substrate of this enzyme *in vivo*. However, from the analysis of the *in vitro* action of D-enzyme on higher molecular weight substrates, amylose or amylopectin, the following new evidence about the action of D-enzyme was obtained. First, high molecular weight amylose and amylopectin can be serve as donor and acceptor. Second, it catalyses the intra-molecular transglycosylation (cyclisation reaction) of amylose and amylopectin to produce cyclic α-1,4-glucan or branched glucan with cyclic structure. Third, very long α-1,4-glucan or even highly branched glucan can be transferred by the enzyme. Fourth, it catalyses the transglycosidic linearisation reaction of cyclic α-1,4-glucan in the presence of acceptor molecule. Additionally, the results strongly suggest that D-enzyme may catalyse the inter-molecular transglycosylation (disproportionating reaction) of amylose or amylopectin, by transferring not only maltooligosaccharide units but also long α-1,4-glucans or highly branched glucan units (Figure 4.18B). Although this disproportionating reaction on high molecular weight starch cannot be

easily detected, it is possible that the disproportionating reaction can occur more frequently than the cyclisation reaction.

These observations therefore suggest numerous possibilities for the function of D-enzyme *in vivo*. First, it may be involved in starch breakdown to convert small oligosaccharides by disproportionation into larger molecules upon which starch phosphorylase can act, as has been previously proposed (Lee and Whelan, 1971; Lin and Preiss, 1988). Amylomaltase has this function in *E. coli*. Second, it may be involved in starch breakdown by generating highly soluble, non-reducing, cyclic glucans which can serve as substrates for amylases, debranching enzyme or starch phosphorylase. It has until now been believed that hydrolysis and phosphorolysis are the only two ways by which starch is broken down, but the present results suggest that the transglycosidic breakdown of starch to produce soluble cyclic glucans should be considered as another possible route. Third, D-enzyme may synthesise cyclic α-1,4-glucan or other cyclic glucans for a function other than starch breakdown. Fourth, it may change the structure of starch molecules by modifying chain lengths in amylose or amylopectin outer chains through its disproportionation reaction. Fifth, it could potentially change the cluster structure of amylopectin or starch grain architecture either during starch grain synthesis or breakdown by transferring highly branched cluster units.

Chapter 5

Analysis of D-enzyme gene expression in potato plants

5.1 Introduction and aims

Analysis of the regulation of gene expression sometimes provides useful information to help understand the role of an enzyme *in vivo*. The expression of genes for starch metabolic enzymes seems to be under developmental and metabolic controls. The mRNAs for genes encoding enzymes for starch synthesis seem to accumulate under circumstances when and where starch biosynthesis is active (in developing seeds or tubers and in illuminated or sucrose-treated leaves) but decline in amount under conditions when starch is broken down (germinating seeds, or sprouting tubers). These enzymes include AGPase small subunit (Müller-Röber *et al.*, 1990), GBSS (Visser *et al.*, 1989) and BE (Koßmann *et al.*, 1991). An activation of gene transcription by sucrose is implied from studies with the GUS reporter gene (Visser *et al.*, 1991). On the other hand, the genes for starch degradation may be differently controlled. For example, Yu *et al.* (1991) showed that α-amylase gene expression in cultured rice cells is induced by carbohydrate starvation and repressed by carbohydrate added to the media. From these results, the expression of genes for enzymes for starch synthesis and those for starch degradation seem to be controlled in opposite fashion.

D-enzyme has been believed to be involved in starch breakdown, but other roles in starch synthesis or in construction of starch structure are also possible as described in Chapter 3. It has been reported that D-enzyme activity is present in leaves and in several starch accumulating storage organs. However, nothing is known about how D-enzyme activity is regulated during organ development or in leaves by light. In order to investigate the role of D-enzyme in plant starch metabolism, it is important to understand how D-enzyme activity and its gene expression is regulated in plants. The isolation of a cDNA for D-enzyme (Chapter 3) and the antibody raised against recombinant D-enzyme polypeptide (Chapter 3) enable the study of D-enzyme synthesis either at the RNA or protein level. The relationship of D-enzyme synthesis with other enzymes of starch metabolism, or with amount of starch accumulated will provide useful information when thinking about the role of this enzyme in starch metabolism. Furthermore the genomic DNA clone isolated in Chapter 3 makes it possible to construct D-enzyme promoter-GUS reporter gene fusion and to analyse the GUS gene expression in transgenic potato plants, in which case the expression of the GUS reporter gene can be measured easily either quantitatively or histochemically. These experiments should provide more information about the regulation of D-enzyme gene expression.

5.2 Analysis of D-enzyme gene expression at the RNA level

In order to investigate the control of D-enzyme gene expression at the RNA level in potato plants, equal amounts of total RNA isolated from a range of organs were analysed by gel electrophoresis, blotting and hybridisation with the D-enzyme cDNA probe. These results (Figure 5.1, DPE) show that D-enzyme RNA is detected in leaves, petioles, stems, roots and stolons, but is most abundant in developing and mature tubers (Figure 5.1, DPE, lanes 6 and 7, respectively), which are accumulating starch. Interestingly, the level of expression is very low or almost zero in tubers stored in the cold room for six months (Figure 5.1, DPE, lane 9), or sprouting tubers (Figure 5.1, DPE, lane 10), which are expected to be degrading accumulated starch. To compare this expression pattern with genes of other starch metabolic enzymes, this RNA blot was hybridised with plastidic starch phosphorylase and granule-bound starch synthase cDNA probes. These genes are chosen as marker genes for starch degradation and starch synthesis, since plastidic starch phosphorylase is generally believed to be involved in starch breakdown, and granule-bound starch synthase is an essential enzyme in starch synthesis. The expression patterns of these genes were very similar to that of D-enzyme (Figure 5.1, PSPP and GBSS).

Leaves were next chosen as a simple experimental system to investigate factors which may regulate D-enzyme gene expression. Leaves were removed after the dark period, then incubated on different sugars, either in the light or the dark for a further 24 h. RNA gel blot analysis of equal amounts of total RNA from each (Figure 5.2, DPE) show that light alone causes a significant increase in the amount of D-enzyme RNA and that this is further increased by sucrose or glucose, but not by mannitol. Sucrose and glucose can also cause an increase in amount in the dark. Thus, light and metabolisable sugars appear to show an additive effect in stimulating D-enzyme RNA accumulation in potato leaves. The amount of RNA correlates well with the amount of starch revealed in each leaf by iodine staining (Figure 5.2, STARCH). Similar patterns of induction of gene expression were also observed in plastidic starch phosphorylase and granule-bound starch synthase genes (Figure 5.2, PSP and GBSS).

In order to examine the induction pattern of expression of these genes more carefully, a similar experiment was carried out by using different concentrations of sucrose. Leaves were removed after the dark period, then incubated on different concentrations of sucrose, either in the light or the dark for a further 24 h. Equal amounts of total RNA from each were blotted and hybridised with these three cDNA

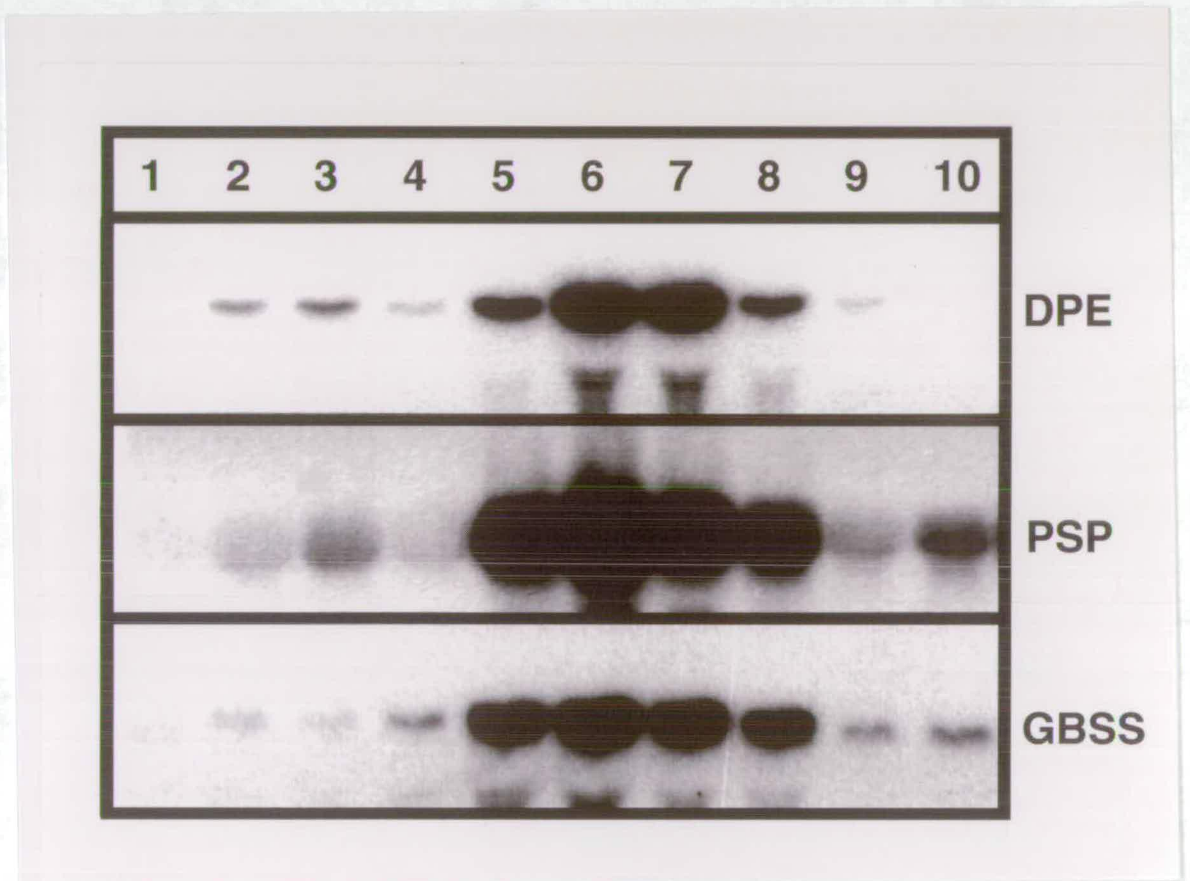


Figure 5.1 Northern blot analysis of the organ specific expression patterns of D-enzyme (DPE), plastidic starch phosphorylase (PSP) and granule-bound starch synthase (GBSS) genes.

Total RNA (20 µg) from the following organs were loaded in each lane and probed with ^{32}P-labelled cDNAs for each enzyme. Lane 1, mature leaves; lane 2, petioles; lane 3, stems; lane 4, roots; lane 5, stolons; lane 6, developing tubers; lane 7, mature tubers; lane 8 and 9, mature tubers stored in cold room (4 °C) for 2 and 6 months; lane 10, sprouting tubers.

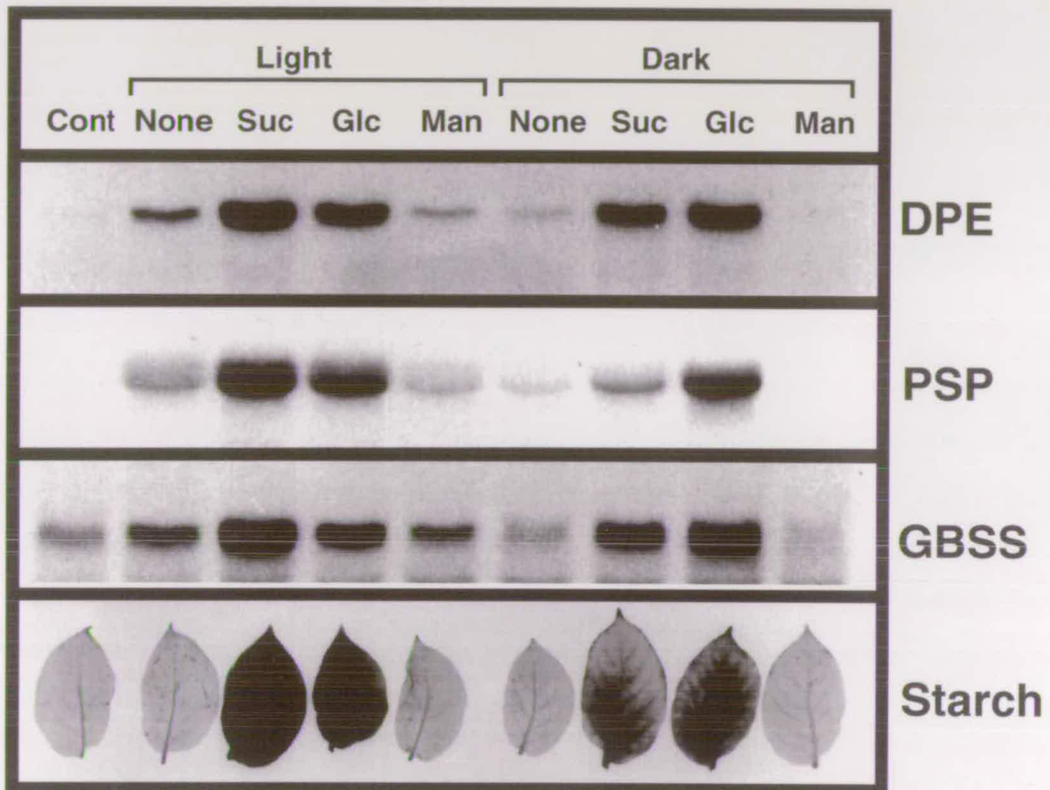


Figure 5.2 Induction of the expression of D-enzyme (DPE), plastidic starch phosphorylase (PSP) and granule-bound starch synthase (GBSS) genes, and starch accumulation (STARCH) in detached leaves by light and carbohydrates.

Total RNA (15 µg) from each of the following samples was hybridised to ^{32}P-labelled cDNA probes for each enzyme. Starch was stained with iodine by the method described in section 2.4.10. Cont, leaves directly harvested at the end of the dark period; in all other lanes leaves were harvested after a further 24 h incubation in the light or dark as shown. Incubation medium was MS medium (None), 20 mM sucrose in MS medium (Suc), 20 mM glucose in MS medium (Glc) or 20 mM mannitol in MS medium (Man).

probes. However, no significant difference was observed among the expression patterns of these three genes. Levels of induction of these genes were greater in higher concentration of sucrose and in the light (Figure 5.3).

5.3 Analysis of D-enzyme gene expression at the protein level

Control of D-enzyme gene expression was next analysed at the protein level. Soluble protein was extracted from the same organ used in northern blot analysis (Figure 5.1), and the amount of D-enzyme protein was analysed by western blot experiment using anti-D-enzyme antisera. D-enzyme protein is detected in leaves, petioles, stems, roots and stolons, but is most abundant in tubers. While the amount of D-enzyme mRNA was declined in stored or sprouting tubers, the amount of D-enzyme protein in these conditions seemed to be unchanged (Figure 5.4, A). The effect of light and carbohydrate on the amount of D-enzyme protein level in detached leaves was next examined. Soluble protein was extracted from each leaflet used in the northern blot experiment and equal amounts of protein from each was analysed by western blot analysis. The amount of D-enzyme seemed to be changed little even in the condition when D-enzyme RNA was induced (Figure 5.4, B).

5.4 Analysis of D-enzyme gene expression using a GUS reporter gene

The analysis of D-enzyme gene expression using the GUS reporter gene system was attempted. 3.0 kb of genomic DNA sequence (containing 5' flanking sequence and coding sequence corresponding to the first 34 amino acids of D-enzyme) was inserted into the *Xba* I and *Hind* III site of plasmid pBI-101, which contained necessary genes and sequences for *Agrobacterium* mediated transformation, so that D-enzyme gene was translationally fused to the GUS reporter gene. The resultant plasmid pBID-GUS (Figure 5.5) and pBI-121 (CaMV 35S promoter fused to GUS) were introduced into potato plants via *Agrobacterium* mediated transformation. Stem cuttings of potato plant, cv May Queen, were transformed with these two gene constructs as described in section 2.10. Shoots grown on shooting medium containing kanamycin were rooted twice on rooting medium containing kanamycin to confirm their kanamycin resistance. Four and two kanamycin resistant regenerated plants were obtained from plasmid pBID-GUS and pBI-121, respectively. Regenerated plants were transferred to soil and grown in the green house.

GUS activity either in leaves or in tubers was first examined in all 6 transformants (table 5.1). Very high GUS activity was obtained in tubers of all transgenic plants carrying the D-enzyme gene promoter, but the GUS activity was

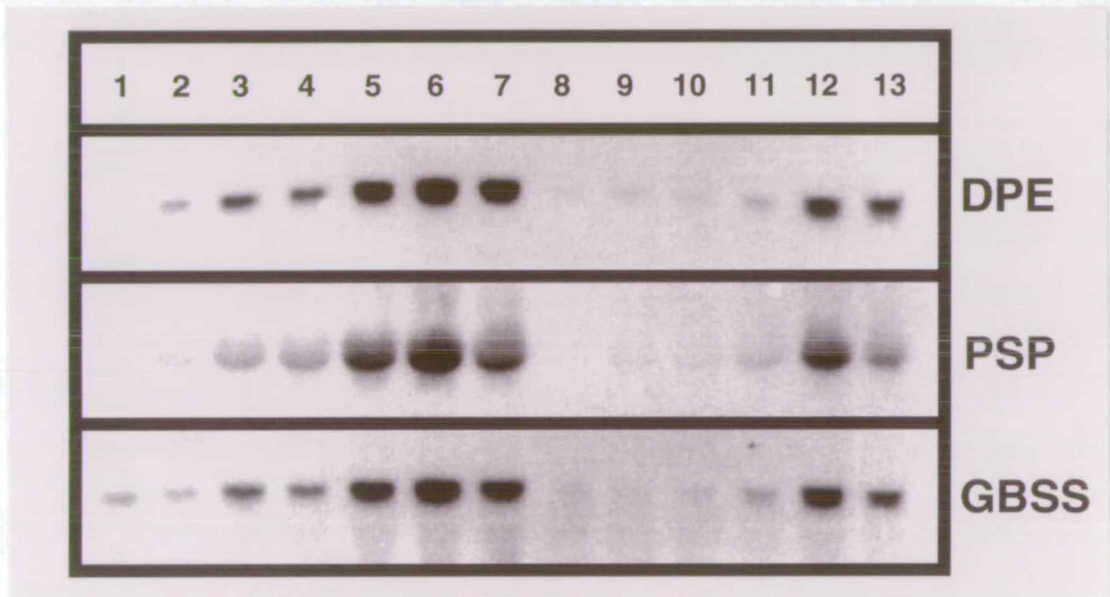


Figure 5.3 Induction of the expression of D-enzyme (DPE), plastidic starch phosphorylase (PSP) and granule-bound starch synthase (GBSS) genes in detached leaves by sucrose

Total RNA (15 μg) of each of the following samples was hybridised to ^{32}P-labelled cDNA probes for each enzyme. Lane 1, leaves directly harvested at the end of the dark period; lanes 2-7, leaves harvested after further 24 h incubation in the light in MS medium containing no, 0.5, 3, 8, 16, 32 mM sucrose, respectively; lanes 8-13, same as lane 2-7, but incubated in the dark.

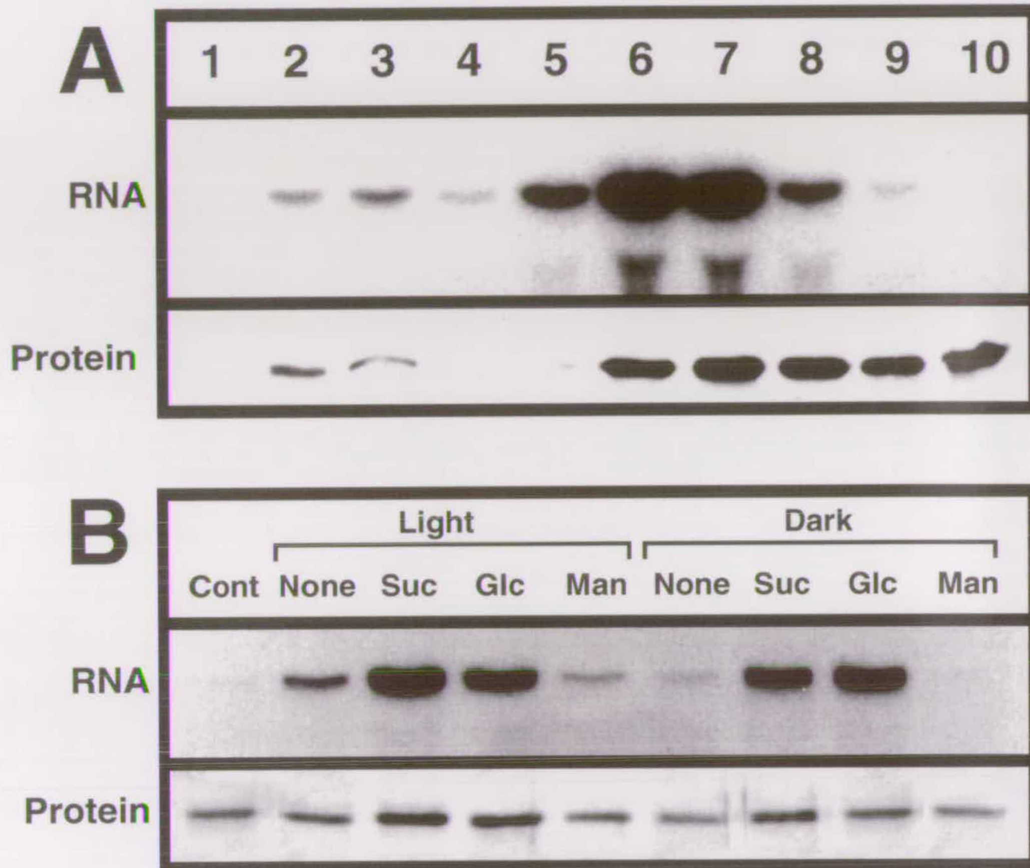


Figure 5.4 Northern and western blot analysis of D-enzyme gene expression in different organs (A) , and in detached leaves (B).
(A) Total RNA (20 µg) from each organs were loaded in each lane and probed with ^{32}P-labelled cDNAs for D-enzyme. Total protein (20 µg) from each organ were separated by SDS-PAGE, blotted onto membrane and probed with D-enzyme antiserum. Lane 1, leaves; lane 2, petioles; lane 3, stems; lane 4, roots; lane 5, stolons; lane 6, developing tubers; lane 7, mature tubers; lane 8 and 9, mature tubers stored in cold room (4 °C) for 2 and 6 months; lane 10, sprouting tubers. (B) Total RNA (15 µg) of each of the following samples was hybridised to ^{32}P-labelled cDNA probes for D-enzyme. Total protein (70 µg) from each sample was separated by SDS-PAGE, blotted onto membrane and probed with D-enzyme antiserum. Cont, leaves directly harvested at the end of the dark period; in all other lanes leaves were harvested after a further 24 h incubation in the light or dark as shown. Incubation medium was MS medium (None), 20 mM sucrose in MS medium (Suc), 20 mM glucose in MS medium (Glc) or 20 mM mannitol in MS medium (Man).

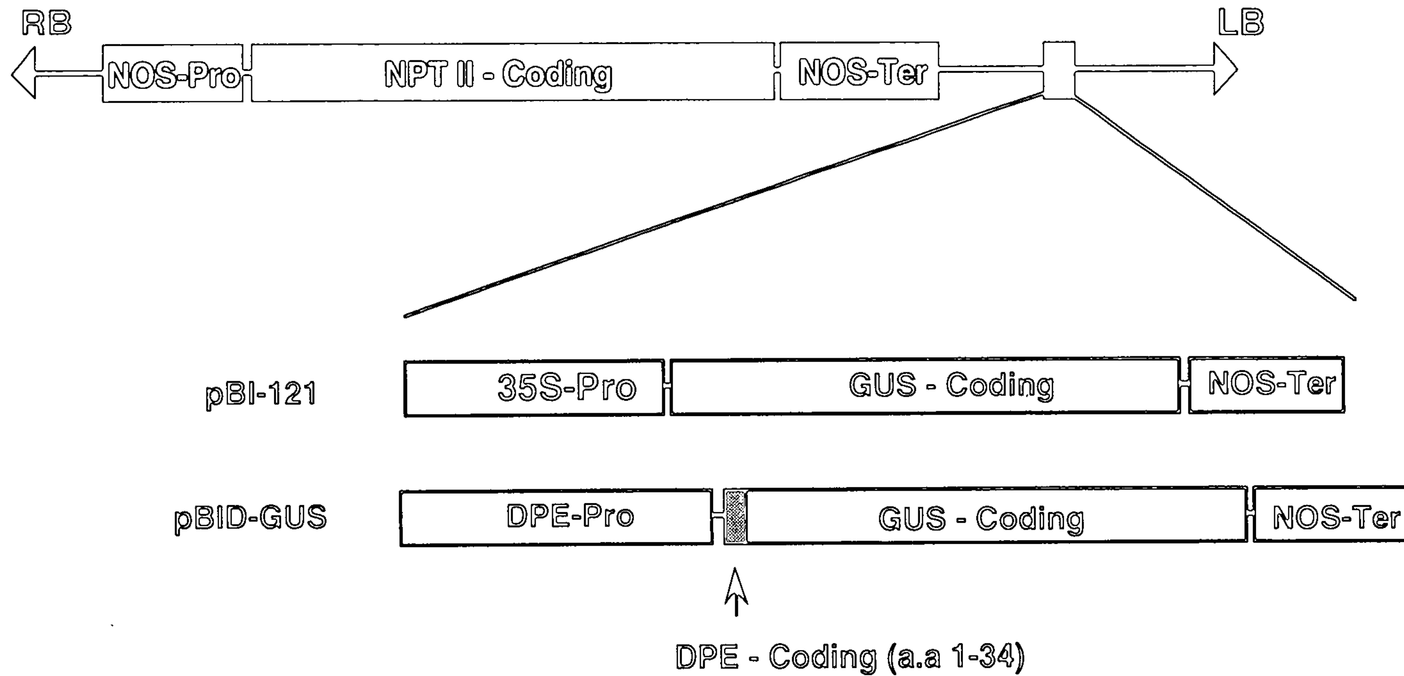


Figure 5.5 Structures of plant transformation vectors
The 3.0 kb *Hind* III - *Xba* I fragment of pGDPE-1 (Figure 3.9) was introduced into the same restriction sites of pBI101 (Clontech) to make a chimeric D-enzyme-GUS fusion gene expression vector, pBID-GUS. RB, T-DNA right border; LB, T-DNA left border; NOS-Pro, nopaline synthase gene promoter sequence; NOS-Ter, nopaline synthase gene terminator sequence; NPT II-Coding, neomycin phosphotransferase-II coding sequence from Tn5; GUS-Coding, β-glucuronidase coding sequence; DPE-Pro, D-enzyme putative promoter sequence; CaMV35S. cauliflower mosaic virus 35S promoter sequence; DPE-Coding, D-enzyme coding sequence. Not drawn to scale.

Table. 5.1 Glucuronidase activity in potato plants transformed with pBID-GUS and pBI-121.

Transformant	Leaf	Tuber
G101	15.5	18110.5
G102	17.2	7039.7
G201	11.8	5201.3
G202	19.6	2881.2
121-101	215.8	295.4
121-102	366.5	131.6

Total protein was extracted from leaves and tubers of each independent transformant and assayed for GUS activity.
GUS activity is expressed as pmol-MU / min / mg-protein.
The values are those of a typical experiment.

only a trace in leaves. This tuber specific expression pattern was found only in transgenics with the D-enzyme gene promoter but not in those with the CaMV35S promoter. The variation in GUS activity in the same construct might be the result of position effect or different transgene copy number (not investigated).

Transformant G101 which showed the highest GUS activity of the four transgenic plants carrying the D-enzyme gene promoter was used for further characterisation. GUS activity in several organs was tested to examine whether the organ specificity of D-enzyme gene expression found in northern blot analysis (Figure 5.1) was conserved in this chimeric construct (Figure 5.6). Similar organ specificity found in GUS activity strongly indicated that the genetic information for developmental regulation of D-enzyme gene expression was located within this 3.0 kb sequence.

5.5 Discussion

It could be expected that genes for starch synthetic enzymes and those for starch breakdown enzymes would show different responses to the same signal, eg. amount of available sucrose. It is true to some extent, because an increasing number of genes encoding enzymes of starch synthesis have been shown to respond to sucrose, resulting in an increase in amounts of mRNA and enzyme. However α-amylase gene expression in cultured rice cells is repressed by available carbohydrates (sucrose, glucose or fructose), but is induced by carbohydrate starvation. As shown in Figure 5.2, D-enzyme mRNA was dramatically increased when detached leaves were incubated with sucrose. Other results also suggested that D-enzyme mRNA accumulates under circumstances when starch biosynthesis is most active (in developing tubers and in illuminated leaves) but declines in amount under conditions when starch is broken down (cold-stored tubers and sprouting tubers). Additionally similar expression patterns at the RNA level (organ specificity and carbohydrate induction) are also reported for the starch synthetic enzymes, BE (Koßmann *et al.*, 1991) and AGPase small subunit (Müller-Röber *et al.*, 1990). These observations appear to contradict the widely held view that D-enzyme is involved in starch breakdown, and may suggest a function in starch synthesis. However, the gene encoding potato plastidic starch phosphorylase also shows a similar pattern of expression. Induction of starch phosphorylase gene during starch synthesis was also reported (St-Pierre and Brisson, 1995). Although plastidic starch phosphorylase is generally believed to be involved in starch breakdown, the role of this enzyme *in vivo*

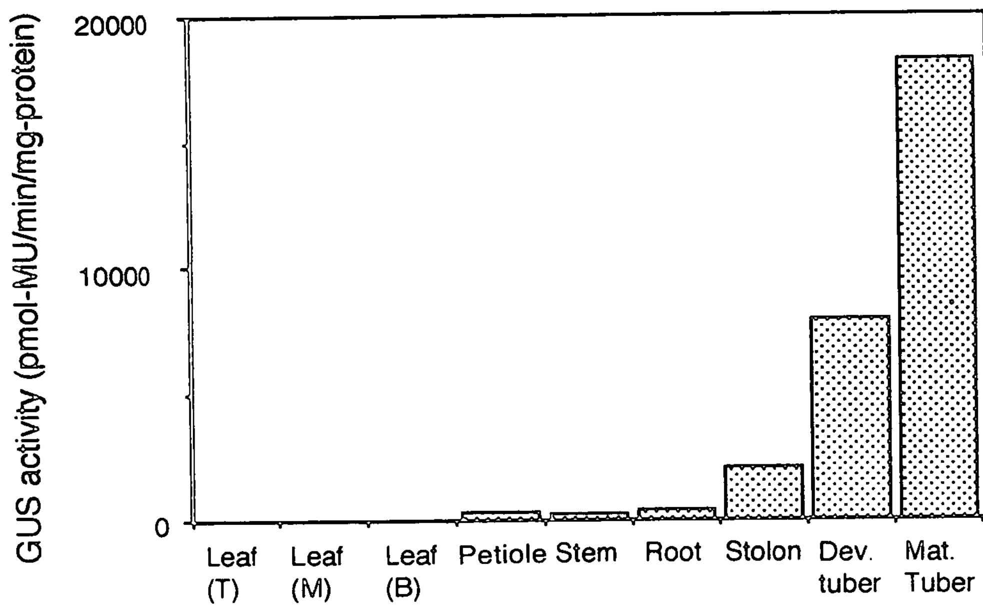


Figure 5.6 Glucuronidase activity in each organ of transgenic potato line G101.
Total protein was extracted from each organ and assayed for GUS activity. (T), (M) and (B), leaves from top, middle and bottom section, respectively. Dev., developing tubers; Mat, mature tubers. The values are those of a typical experiment.

is still questionable. The results obtained here may suggest that plastidic starch phosphorylase could be involved in starch synthesis.

The results presented here are not sufficient to suggest the role of D-enzyme *in vivo*, but they provide interesting information about the regulation of D-enzyme gene expression. D-enzyme gene expression seemed to be under developmental and metabolic control at the transcriptional level in potato plants. However, the analysis of gene expression at the protein level suggested that the amount of RNA did not always indicate the amount of protein. The abundant mRNA found in mature tuber was greatly decreased during storage and sprouting, however the amount of protein did not change. In addition, the amount of mRNA in leaf was increased dramatically in 24 h in response to added sucrose, but the amount of protein seemed to be constant. These results may suggest that D-enzyme gene expression is controlled at both transcriptional and posttranscriptional levels.

The transgenic potato plants transformed with a chimeric gene (D-enzyme gene promoter sequence translationally fused to GUS reporter gene), should be extremely useful for further investigation of the regulation of D-enzyme gene expression. Preliminary analysis of GUS activity in several organs indicated that the 3.0 kb promoter sequence employed seems to contain necessary genetic information for developmental regulation of D-enzyme gene expression. At the moment, it is not known whether this promoter sequence also contains genetic information for transcriptional control by metabolites (sucrose) and posttranscriptional control, but such experiments can now be carried out.

Chapter 6

Production and analysis of transgenic plants with altered levels of D-enzyme activity

6.1 Introduction and aims

The analysis of mutants provides valuable information in understanding biological processes and systems. It is true in plant starch metabolism, because the understanding so far achieved in the plant starch synthetic pathway owes much to such information, as described in Chapter 2. It has been believed that D-enzyme is involved in starch breakdown, to convert short oligosaccharides into longer chains upon which starch phosphorylase can act. However, as described in Chapter 4, other roles of this enzyme in starch metabolism are possible, since D-enzyme catalyses several new reactions on high molecular weight starch. It is obvious that an important approach to investigate the role of D-enzyme is to analyse D-enzyme deficient mutants. However no mutant for D-enzyme has been isolated from any higher plant, and it is also difficult to screen mutants since we do not know the phenotypic change caused by D-enzyme inactivation.

It has been shown that antisense RNA transcribed from an antisense copy of the gene inhibits the expression of a target gene and can mimic mutations in prokaryotic and eukaryotic cells. Although the mechanism of inhibition is not clearly understood, numbers of antisense plants have been produced and used to investigate the function of unknown genes or to manipulate metabolism. Almost complete inhibition of AGPase synthesis and concomitant abolition of starch formation in tubers was achieved by introducing an antisense AGPase B subunit gene (Müller-Röber *et al.*, 1992). Transgenic potato plants carrying an antisense GBSS gene also showed decreased enzyme activity (30 to 0 % of wild type) and decreased amylose content (Visser *et al.*, 1991).

The isolation of the cDNA for D-enzyme as described in Chapter 3 enables transgenic plants to be produced by introducing a D-enzyme gene either in sense or antisense orientation relative to an appropriate promoter sequence. The levels of D-enzyme gene expression can be examined by measuring the amount of mRNA, amount of D-enzyme protein and D-enzyme activity. The selected transformants can be used for further analysis to determine the effect of altered levels of D-enzyme on plant growth, tuber development or starch metabolism.

6.2 Production of transgenic potato plants with altered levels of D-enzyme activity

6.2.1 Construction of transformation vectors and transfer to potato plants

Potato plants having decreased or increased levels of D-enzyme may provide important information about the function of D-enzyme *in vivo*. Thus several gene vectors which were expected to increase or inhibit the synthesis of D-enzyme were constructed (Figure 6.1). pBIC-102F and pBIC-102R are designed to synthesise sense and antisense RNA, respectively, in most of plant tissues, from D-enzyme cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter. pBIP-102F and pBIP-102R were designed to synthesise sense and antisense RNA, respectively, in tubers, from D-enzyme cDNA under the control of the patatin promoter. These four genes were constructed in pBI101 plasmid vector (Clontech) which contains the necessary genes and sequences for *Agrobacterium* mediated transformation.

Stem cuttings of potato, cv May Queen, were transformed with these four gene constructs as described in section 2.10. Shoots grown on shooting medium containing kanamycin were rooted twice on rooting medium containing kanamycin to confirm their kanamycin resistance. Regenerated plants (T1-1 plants) were transferred to soil and grown in the greenhouse and tubers were harvested (T1-1 tubers). The scheme of growth and analysis of transgenic plants is shown in Figure 6.2.

6.2.2 Screening of transgenic plants for altered D-enzyme activity

One tuber for each independent transformant was used to examine the D-enzyme activity. Results are summarised in Figure 6.3. Various degrees of reduced D-enzyme activity (from 90 to 1 % of wild type) were found in transgenic plants carrying antisense constructs. Greater reduction of D-enzyme activity was achieved with the CaMV 35S promoter than with the patatin promoter, although this was not expected. D-enzyme activity levels of most of the sense transformants were not largely changed from wild type (range within ± 20 % of wild type). However two sense transformants (D103 and D204) showed greatly reduced D-enzyme activity (about 1 % of wild type). Similar inhibition of gene expression by sense transgenes has been reported by several groups and is called 'co-suppression'. Investigation of the

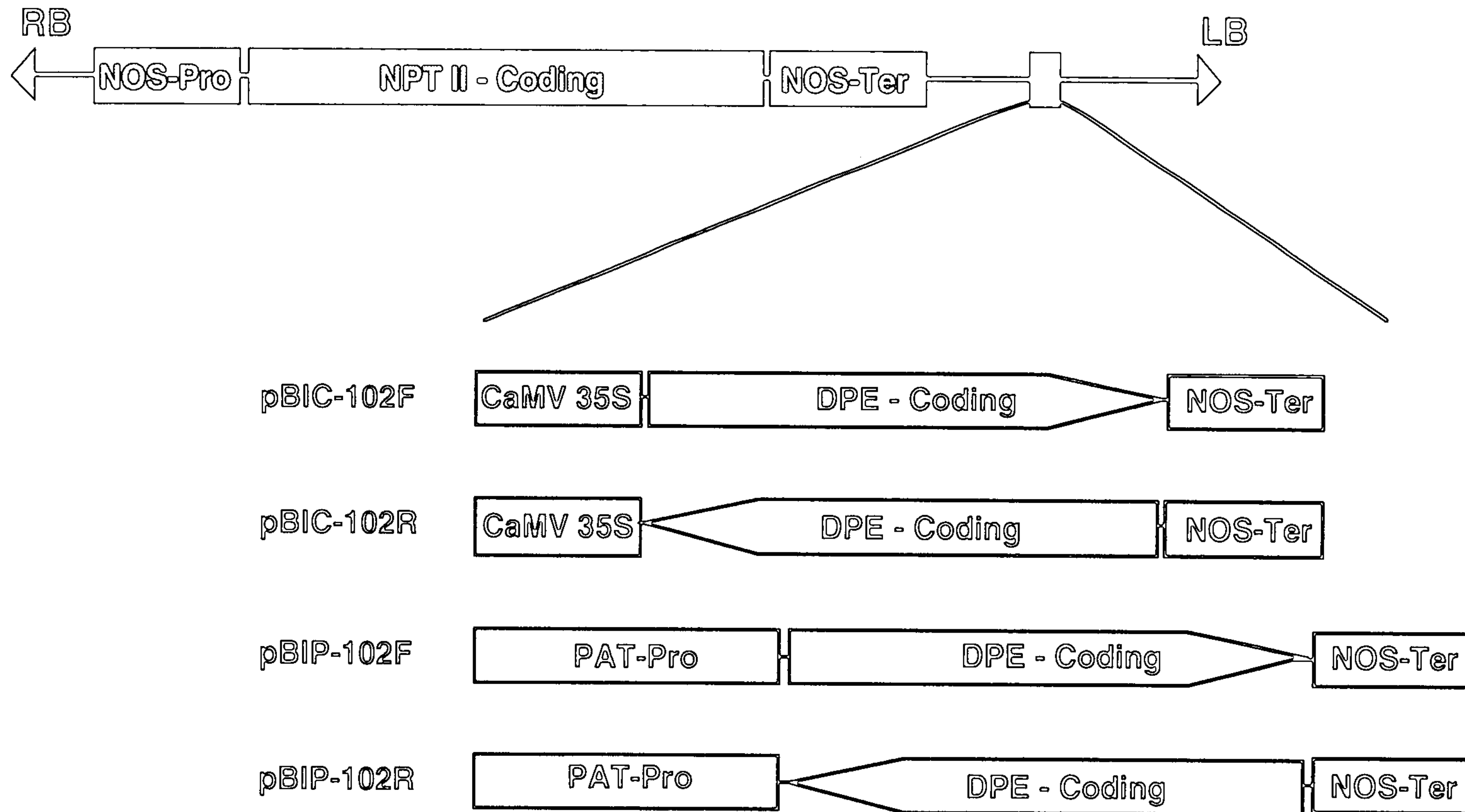


Figure 6.1 Structures of plant transformation vectors
Plant transformation vectors were constructed as described in section 2.10.1. RB, T-DNA right border; LB, T-DNA left border; NOS-Pro, nopaline synthase gene promoter sequence; NOS-Ter, nopaline synthase gene terminator sequence; NPT II-Coding, neomycin phosphotransferase-II coding sequence from Tn5; CaMV 35S. cauliflower mosaic virus 35S promoter sequence; DPE-Coding, D-enzyme coding sequence; PAT-Pro, patatin promoter sequence. The DPE-coding sequence is inserted either in forward (F) or reverse (R) orientation to give sense and antisense expression , respectively.

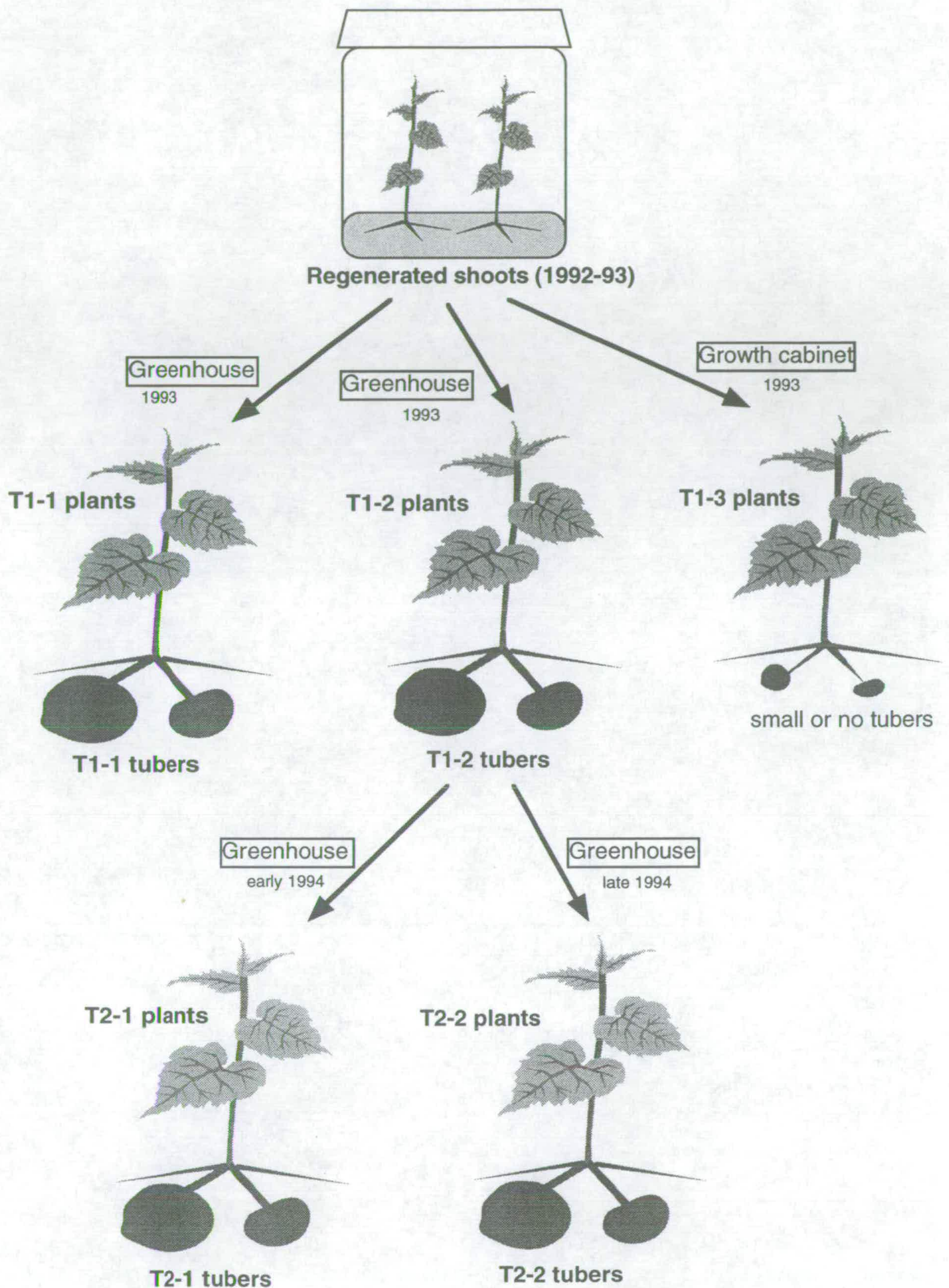


Figure 6.2 The scheme of growth and analysis of transgenic plants
Summary of growth of transgenic potato plants. The growth of T1-1, T1-2, and T1-3 plants were started from regenerated shoots maintained in *in vitro* shoot culture. T2-1 and T2-2 plants were started from T1-2 tubers. T1-3 plants were grown in growth cabinet and the others were grown in greenhouse.

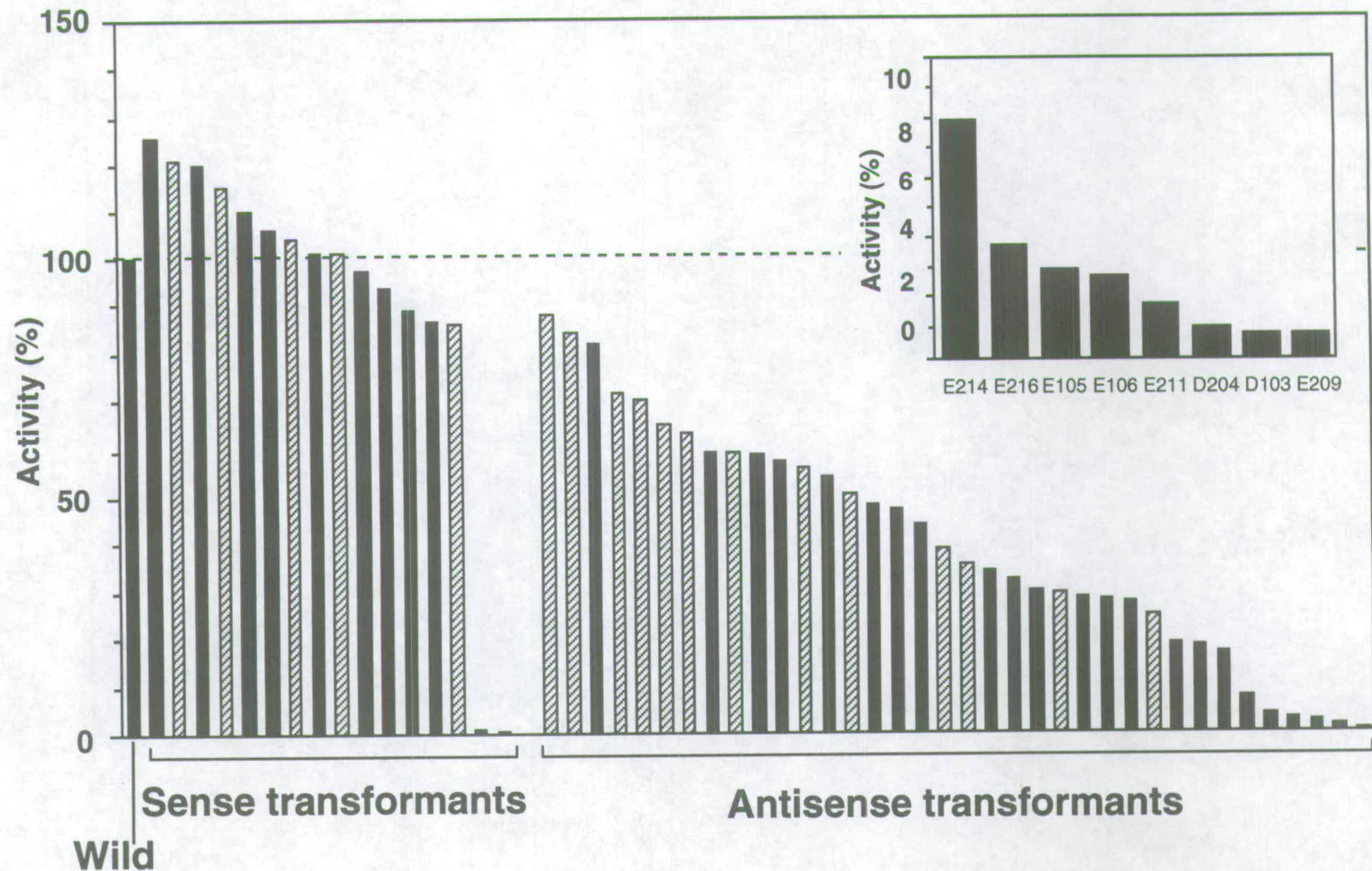


Figure 6.3 D-enzyme activities in tubers of transformed potato plants
One tuber from each independent transformant was used to examine the D-enzyme activity. D-enzyme activities are expressed relative to that of wild type. Solid and hatched bars are plants transformed with 35S promoter and patatin promoter constructs, respectively. The box (inset) gives values for selected transformants with low activity.

mechanism of reduced activity in sense transformants was not carried out in this thesis.

6.2.3 Analysis of D-enzyme gene expression in tubers of transgenic plants

Three antisense transformants with reduced D-enzyme activity (E105,E211 and E209) and wild type were used for further investigation. In order to examine whether the reduced activity of D-enzyme was caused by reduced D-enzyme protein level, tuber extracts (T1-1 tubers, see Figure 6.2) from each plant were subjected to western blot analysis using an antibody for D-enzyme. The strong signal found in wild type could not be detected in the tuber extracts of E211 or E209, but a very faint band was detected in the E105 extract (Figure 6.4B, DPE). However the amount of plastidic starch phosphorylase examined by activity staining of PAGE seems to be constant in wild type and transgenic plants (Figure 6.4B, PSP).

Northern blot analysis was next carried out to examine the level of D-enzyme gene transcripts in tubers. The strong signal found in the wild type tuber could not be detected in the three transgenic plants examined (Figure 6.4C, DPE). The same RNA blot was also probed with a plastidic starch phosphorylase cDNA sequence, but no reduction of transcript level was observed in RNA from antisense plants (Figure 6.4C, PSP). Thus the reduced D-enzyme activity found in the transgenic plants seems to be caused by the specific elimination of D-enzyme mRNA as well as D-enzyme protein.

6.2.4 Analysis of D-enzyme activity and protein in leaves of transgenic plants

D-enzyme is known to be present in potato leaves, although the level is far less than in tubers. Since the antisense gene in these three transformants is controlled by the CaMV 35S promoter, similar reduced D-enzyme activity in leaf tissue can be expected. As shown in Figure 6.5A, D-enzyme activity was also reduced in leaves of these plants, but the level of reduction was less than obtained in tubers. The reduced D-enzyme activity was accompanied by reduced D-enzyme protein as shown in a western blot (Figure 6.5B, DPE), and the reduction seems to be specific to D-enzyme protein since the level of plastidic starch phosphorylase (detected by activity staining) was constant (Figure 6.5B, PSP).

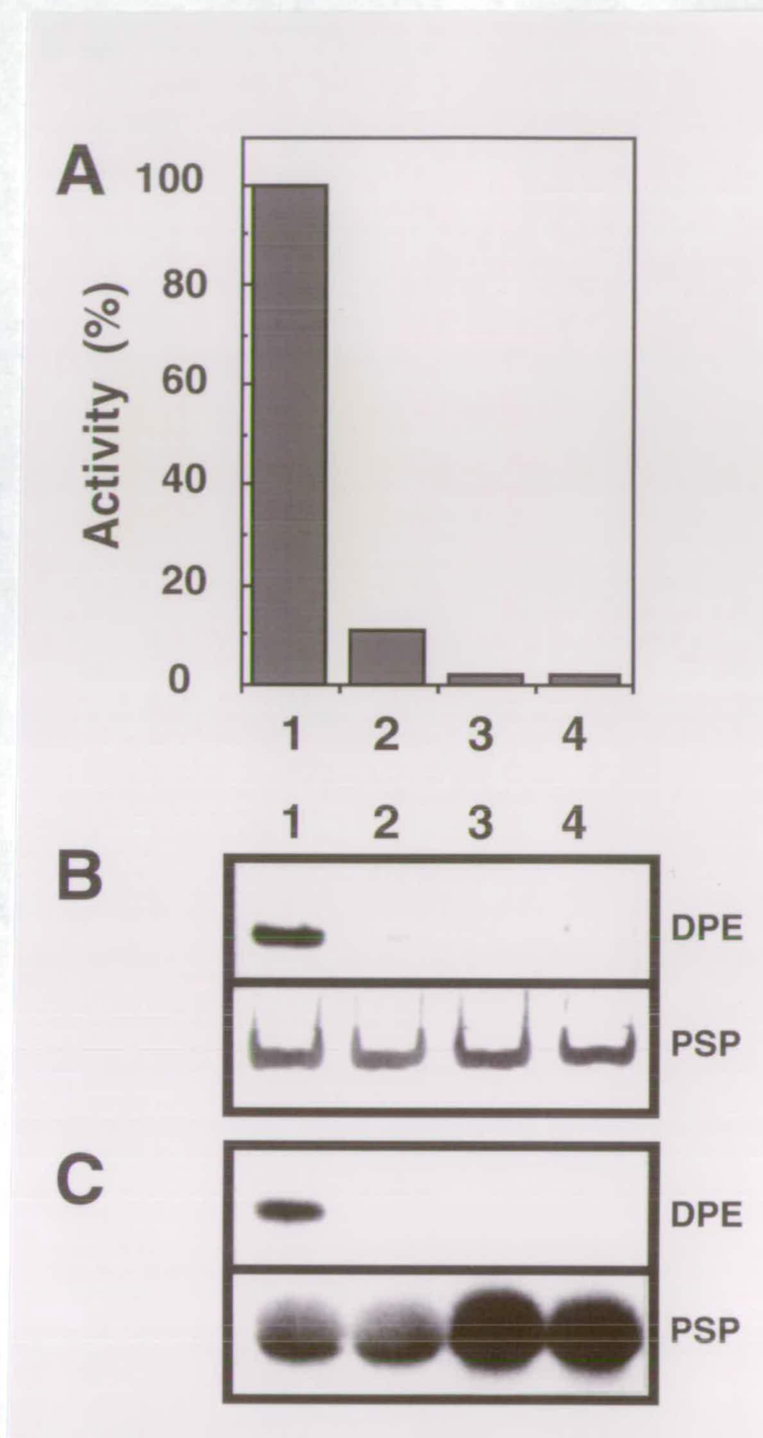


Figure 6.4 Inhibition of D-enzyme gene expression in tubers of transgenic plants
(A) D-enzyme activities are measured as Figure 6.3 and expressed relative to that of wild type. (B) Total protein (20 μg) from each was separated by SDS-PAGE, blotted onto membrane and probed with D-enzyme antiserum (DPE). Total protein (20 μg) from each was separated by native-PAGE containing glycogen and plastidic starch phosphorylase activity is visualised as described in section 2.3.7 (PSP). (C) Total RNA (20 μg) from each was loaded in each lane and probed with ^{32}P-labelled cDNAs for D-enzyme (DPE) and plastidic starch phosphorylase (PSP). Lane 1, wild type; lane 2, E105; lane 3, E211; lane 4, E209.

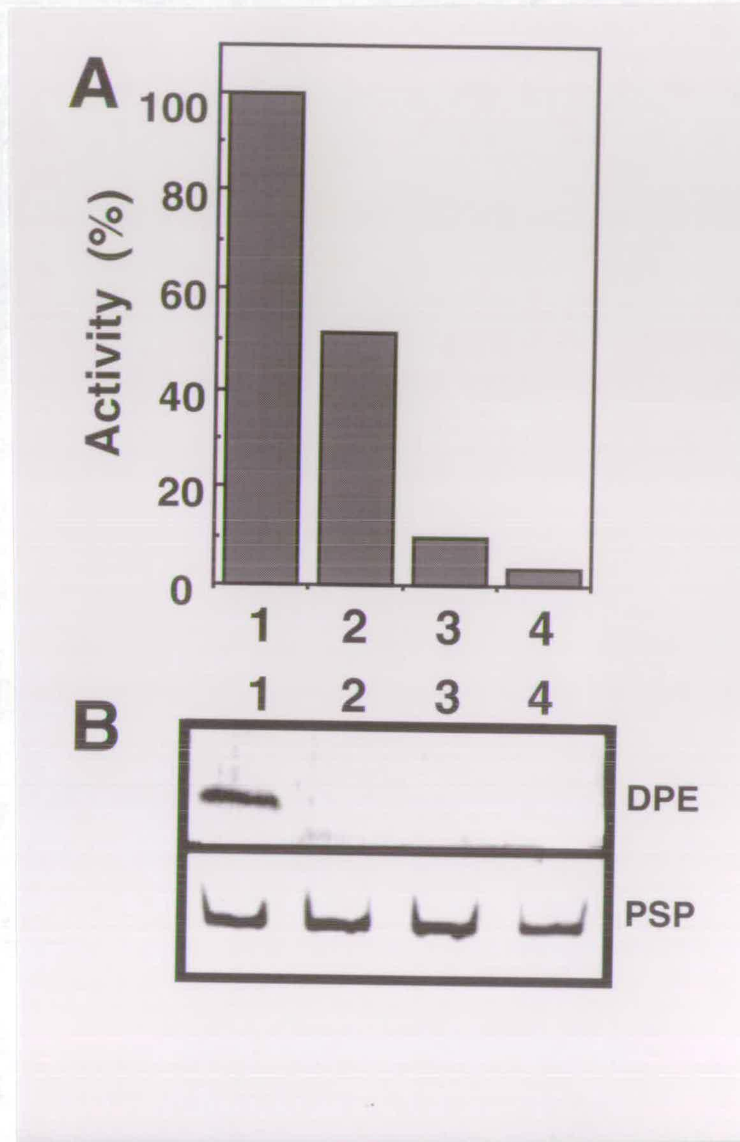


Figure 6.5 Inhibition of D-enzyme gene expression in leaves of transgenic plants
(A) D-enzyme activities in leaves were measured and expressed relative to that of wild type. (B) Total protein (70 μg) from each was separated by SDS-PAGE, blotted onto membrane and probed with D-enzyme antiserum (DPE). Total protein (90 μg) from each was separated by native-PAGE containing glycogen and plastidic starch phosphorylase activity was visualised as described in section 2.3.7 (PSP). Lane 1, wild type; lane 2, E105; lane 3, E211; lane 4, E209.

6.3 Effect of reduced D-enzyme activity on plant growth and tuber development

6.3.1 Production of transgenic tubers

Six transgenic plants with reduced D-enzyme activity (four with antisense (E105, E106, E209, E211) and two with sense (D103, D204) gene constructs) and wild type were maintained by *in vitro* shoot culture as described in section 2.10.3. In order to obtain tubers for further analysis, eight plants for each were transferred to soil and four grown in large pots in a greenhouse (T1-2 plants) and four in small pots in a growth cabinet at 25 °C with 16 h photoperiod at an irradiance of 100 μmol $m^{-2}s^{-1}$ (T1-3 plants). Temperature and lighting in the greenhouse were less rigorously controlled, but could reach 30 °C and 300 μmol $m^{-2}s^{-1}$ on occasions, and plants were less crowded than in the growth cabinet. Tubers were harvested from those grown in the greenhouse (T1-2 tubers), but transgenic plants in the growth cabinet (T1-3 plants) produced very few tubers, or none in some cases. In order to examine the stability of reduced D-enzyme activity and other phenotypic characters found in transgenic plants in the second generation, four T1-2 tubers for each construct were planted in, 1994 and grown in the greenhouse and tubers harvested (T2-1 tubers). Four other T1-2 tubers for each construct were also planted later in, 1994 and grown in the greenhouse and tubers harvested (T2-2 tubers). T2-1 and T2-2 tubers were harvested by Ms Joanna Critchley and Dr. Steven M. Smith, respectively. The scheme of growth of transgenic plants is summarised in Figure 6.2.

6.3.2 D-enzyme activity

D-enzyme activity in tubers was assayed and summarised in Table 6.1 (Assays of T2-1 tubers were carried out by Ms Joanna Critchley). Four transgenic plants (E211, E209, D103, D204) which had less than 2 % of wild type activity in the primary screening experiment using T1-1 tubers, also showed very low (less than 2 % of wild type) D-enzyme activity in other tubers produced from *in vitro* shoots (T1-2), and even in the tubers in the second generations (T2-1). Thus the suppression of D-enzyme gene expression and the resultant low D-enzyme activity seems to be very stable in these four transgenic lines. However, the D-enzyme activity in E105 and E106 was not constant between different cultivation groups. From the activity value obtained in each tuber, this instability seemed to be caused by variations between plants and between tubers within a single plant. For this reason, D-enzyme activity in three different tubers (T2-1) from each of four E105 and E106 plants was analysed, to

Table 6.1 D-enzyme activity in tubers from wild type and transgenic potato plants

Transformant	T1-1	T1-2	T2-1	T2-2
Wild	100	100	100	100
E105	3.0	4.4	20.3	n.t.
E106	2.7	13.3	30.6	n.t.
E211	1.7	1.4	1.7	n.t.
E209	0.8	1.3	1.1	n.t.
D103	0.8	1.1	1.1	n.t.
D204	1.1	0.8	0.6	n.t.

The value is the mean of the values obtained from at least three independent plants.
D-enzyme activities are expressed (as percentage) relative to that of wild type.
n.t., not tested

investigate the variation between plants or tubers. The activity of D-enzyme from each tuber was plotted as shown in Figure 6.6. In E105 and E106, large variation (from almost zero to wild type level) was found between plants and in tubers within a single plant, which is not observed in wild type and E209 plants. Such heterogeneous suppression of gene expression by a transgene has been reported in potato plants having an antisense GBSS gene (Kuipers *et al.*, 1994).

6.3.3 Plant Growth

In order to investigate the effect of reduced D-enzyme activity on plant growth, eight *in vitro* shoots for each were transferred to soil and grown either in the greenhouse or in the growth cabinet. No significant difference was found in the appearance of wild type and transgenic plants grown in the greenhouse. However slow growth was observed in E211, E209 and D103 plants grown in the growth cabinet (Figure 6.7). Great differences were found especially in E209 and D103 plants, which have second and third lowest activity (table 6.1). E209 plants not only grew slower than wild type plants but also showed differences around the shoot apex. E209 plants appeared to produce an unhealthy shoot apex and several side shoots were sometimes produced since the original one was dead (Figure 6.8). D103 grew slower and seemed to have smaller and fewer leaves than wild type. These results may indicate that the effect of reduced D-enzyme activity might be more pronounced in limited light or when plants are grown in small pots.

Plant growth was next analysed using shoots from seed tubers. T1-2 tubers were kept in the dark at room temperature until they produced sprouts, then kept in the cold room until all the tubers had produced strong sprouts. All the sprouts were removed from the seed tubers except the strongest one, then planted in large (10 inch) pots and incubated in the growth cabinet. The height of the plants and the number of leaves were measured during the first few weeks and results plotted in Figure 6.9. Growth of E209 and D103 plants was again apparently slower than that of wild type. The height of these plants was approximately half of wild type, and the number of leaves was about one-third of wild type at all points tested. The plants containing low D-enzyme activity produced flowers later than wild type plants (results not shown) and in E209 and D103 plants, the flower buds sometimes abscised before opening. Once all plants had flowered, they were transferred to the greenhouse to continue growth to maturity.

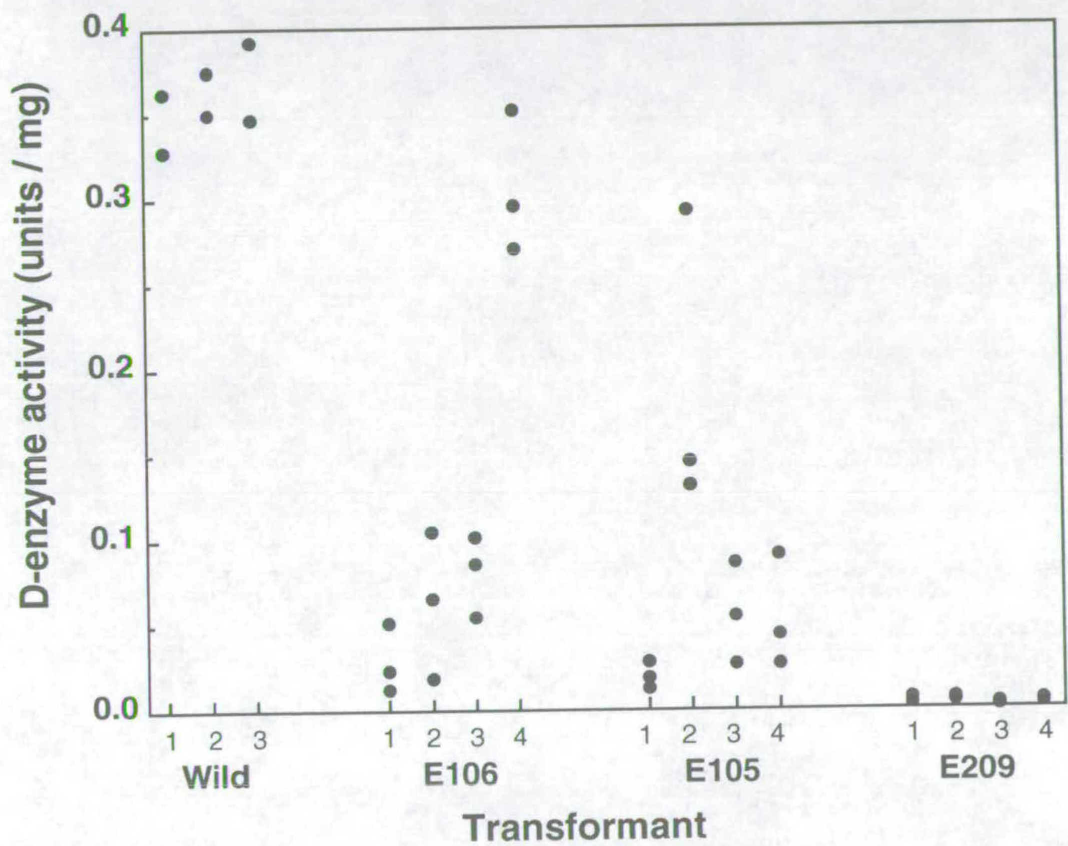


Figure 6.6 Variation of D-enzyme activity between plants and tubers
D-enzyme activity was measured in three different tubers from each of four E209, E105 and E106 plants and in two different tubers from each of three wild type plants.

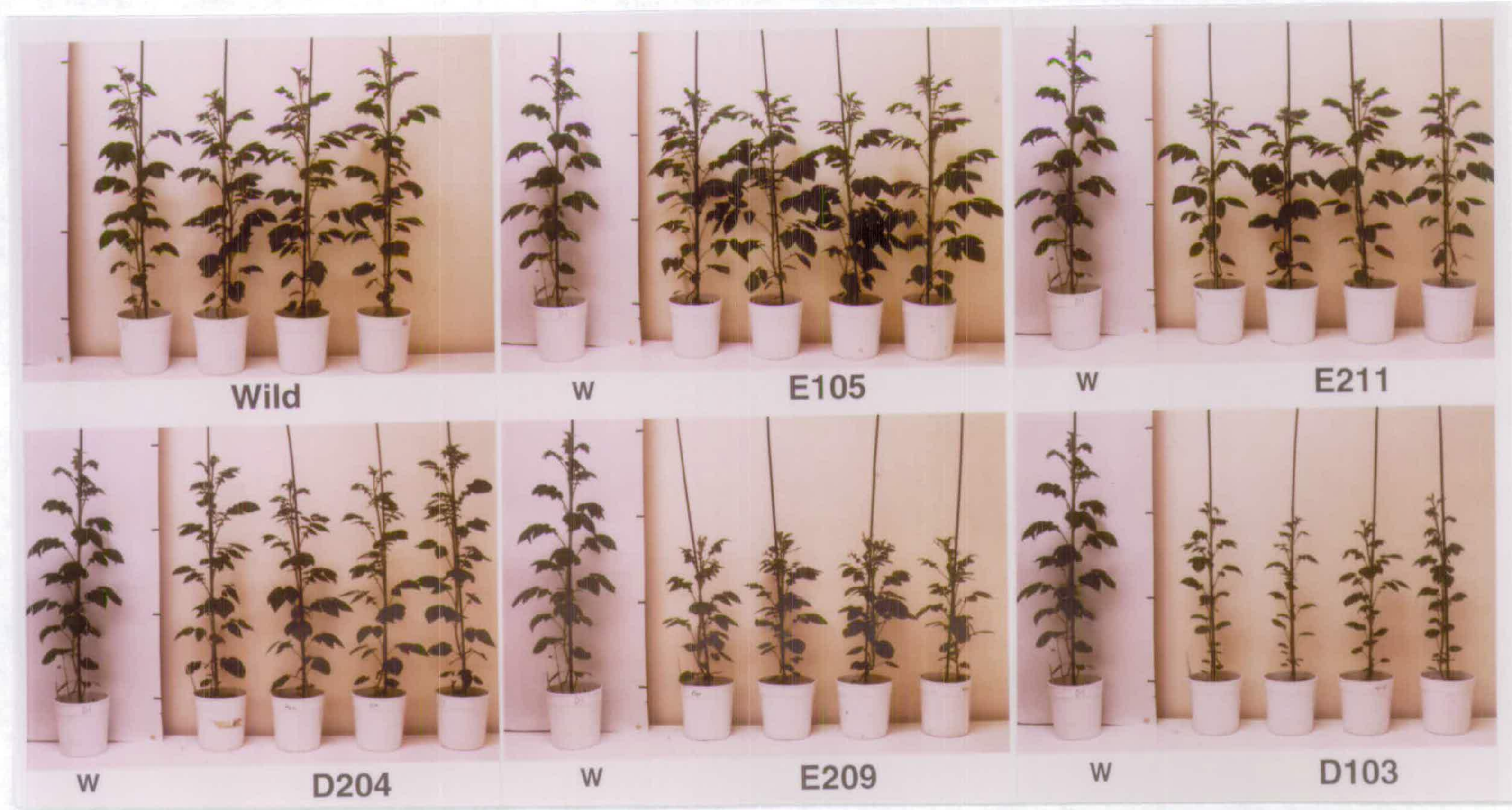


Figure 6.7 Effect of decreased D-enzyme activity on plant growth
Four *in vitro* shoots for six transgenic plants with reduced D-enzyme activity (E105, E209, E211, D103, D204) and wild type were transferred to soil and grown in a growth cabinet at 25 °C with 16 h photoperiod at an irradiance of 100 µmol $m^{-2}s^{-1}$ for 5 weeks, and photographed. W, one wild type plant for comparison.

Wild

E209

Figure 6.8 Effect of decreased D-enzyme activity on the shoot apex. Plants are those shown in Figure 6.6.

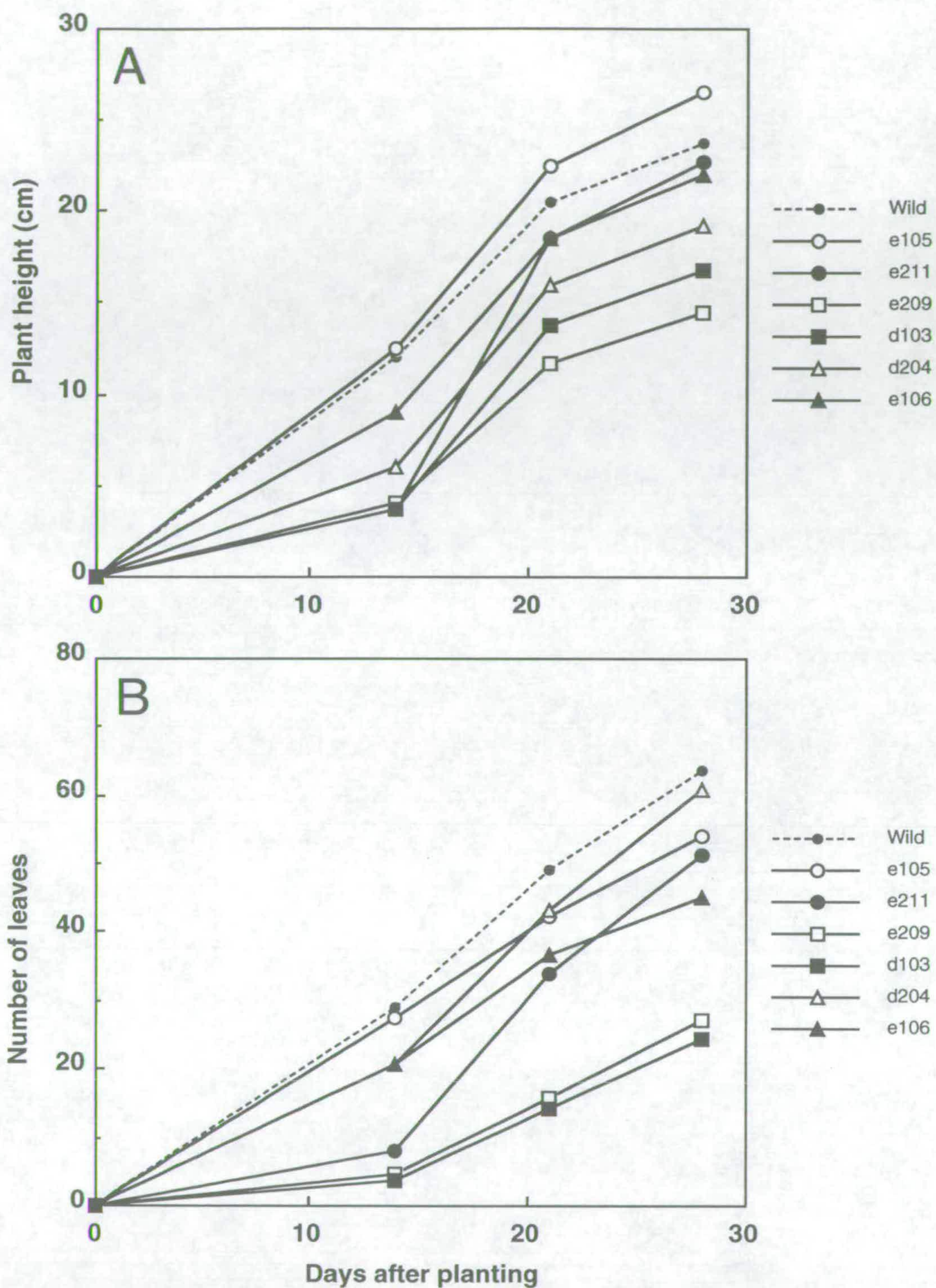


Figure 6.9 Effect of decreased D-enzyme activity on plant height and number of leaves
T1-2 tubers were planted in pots and incubated in the growth cabinet at 25 °C with 16 h photoperiod at an irradiance of 100 μmol $m^{-2}s^{-1}$. The height of the plants (A) and the number of leaves (B) were measured during the initial few weeks. Value are the means of four plants for each point.

6.3.4 Tuber yield

The number of tubers (more than 2 cm in diameter) and total weight of tubers from each plant were measured and are summarised in Tables 6.2 (measurement was carried out by Ms. Joanna Critchley (T2-1) and Dr. Steven M. Smith (T2-2)). The harvested T2-1 tubers are also shown in Figure 6.10.. No significant difference was observed in number of tubers between wild and transgenic plants. There appears to be a reduction in the mass of tubers produced by E209 and D103. These transgenic plants produced less mass of tubers than wild type in all cultivation groups. As cultivation was not carried out under identical conditions, the yield of tubers varies very much even for the same transformant. However the low yield of E209 and D103 relative to wild type seemed to be reproducible in these three different experiments.

Scanning electron microscopy analysis of the cut surface of these tubers was carried out in order to characterise the cells and starch granules (carried out by Edinburgh University, Science Faculty Electron Microscope Facility). The pictures of the cut surfaces of wild type, E105 and D103 tubers are shown in Figure 6.11. No difference was found in size and shape of cells and size, shape and number of starch granules.

6.3.5 Tuber sprouting

The effect of reduced D-enzyme activity on tuber sprouting was next investigated. Harvested T1-2 tubers were kept at room temperature in the dark, and the number of sprouts (longer than 5 mm) and length of each sprout was measured (carried out by Dr. Steven M. Smith). These results are presented in Figures 6.12, 6.13. As shown in Figure 6.12, the first sprout was found at 16 weeks after harvest and all wild type tubers had sprouted at 24 weeks after harvest. However the sprouting of transgenic tubers was slower, with D103 and E209 being particularly slow to produce sprouts. The first sprout was observed 22 weeks after harvest in D103 and E209 tubers. Furthermore, a difference was also found in the growth rate of the sprouts. As shown in Figure 6.13A, total sprout length of each wild type tuber increased to more than 15 cm in 6 weeks, but those of E209 and D103 increased less than 10 cm in 8 weeks. However, the growth rate of individual sprouts was even slower for D103 tubers (Figure 6.13B) because each tuber produced more sprouts than other tubers.

Table 6.2 Numbers and total weight of tubers from wild type and transgenic potato plants

Transformant	Number of tubers			Total weight of tubers					
	T1-2	T2-1	T2-2	T1-2 (g)	T1-2 (%)	T2-1 (g)	T2-1 (%)	T2-2 (g)	T2-2 (%)
Wild	8.0±1.0	14.0±3.0	19.0±8.0	147.3±35.0	100	420.0±122.0	100	312.0±117.0	100
E105	4.5±1.0	10.8±1.3	15.3±8.7	142.5±14.8	96.8	422.5±55.3	100.6	217.4±87.7	69.7
E106	7.5±1.3	7.8±4.2	15.0±1.4	139.5±15.7	93.7	374.1±74.9	89.1	249.5±50.5	80.0
E211	7.7±1.5	11.8±5.0	16.0±6.3	120.2±76.3	81.6	318.6±39.2	75.9	237.7±39.1	76.2
E209	6.0±1.4	12.8±5.6	7.3±1.0	104.6±17.9	71.0	297.4±13.9	70.8	116.3±36.8	37.3
D103	4.0±1.8	11.5±2.1	9.5±1.7	99.2±20.9	67.4	368.5±42.5	87.8	135.3±38.9	43.4
D204	10.0±2.4	11.5±4.7	18.3±1.7	157.4±27.6	106.9	403.1±95.0	96.0	239.8±42.3	76.9

The value is the mean of at least three independent plants.

Figure 6.10 Tubers harvested from wild and transgenic plants
The harvested T2-1 tubers (more than 2 cm in diameter) from each of 4 plants were photographed.

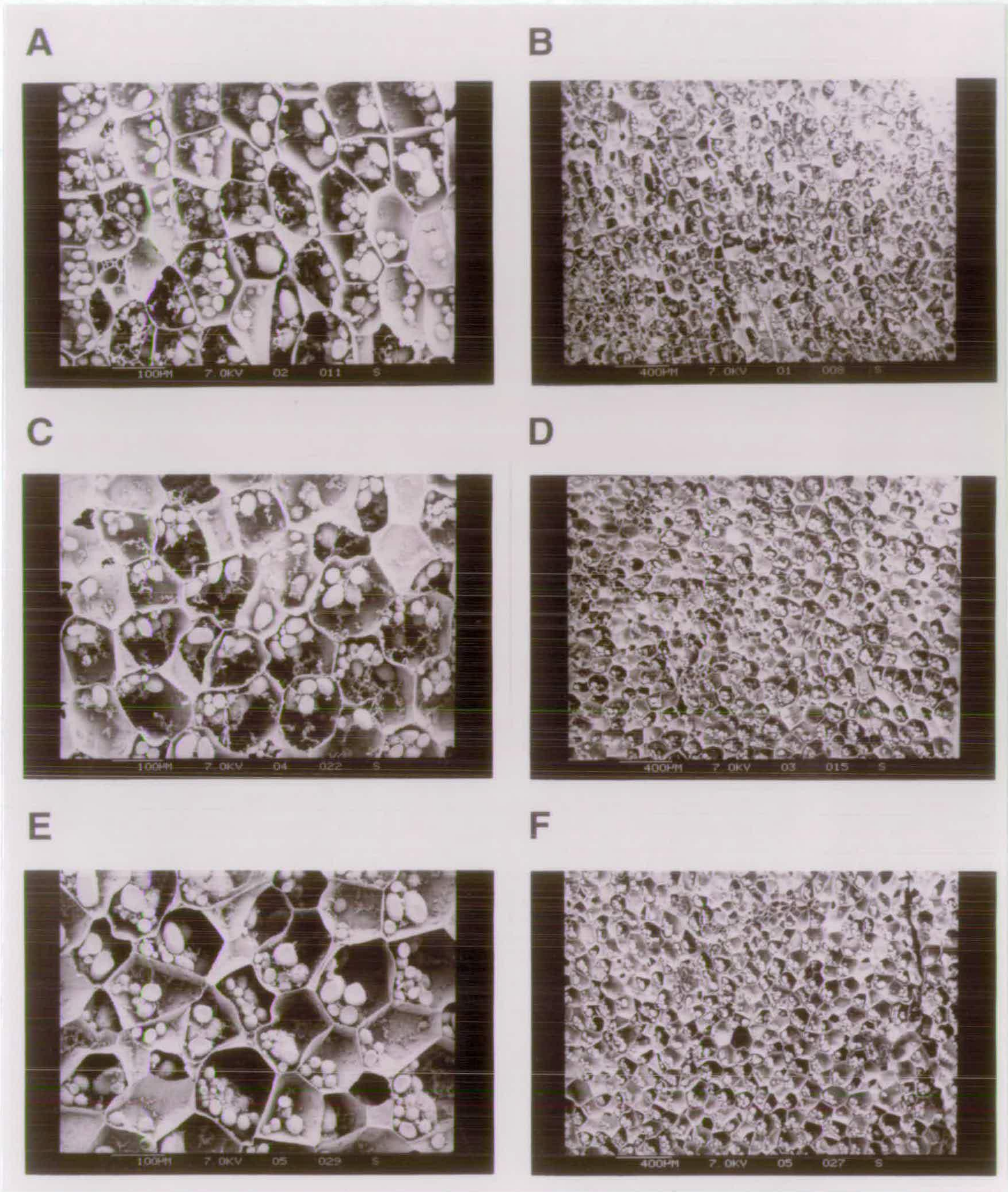


Figure 6.11 SEM analysis of the cut surface of wild and transgenic tubers Scanning electron microscopic analysis of the cut surface of tubers from wild and transgenic plants. A, wild, x100; B, wild, x10; C, E209, x100; D, E209, x10; E, D103, x100; F, D103, x10.

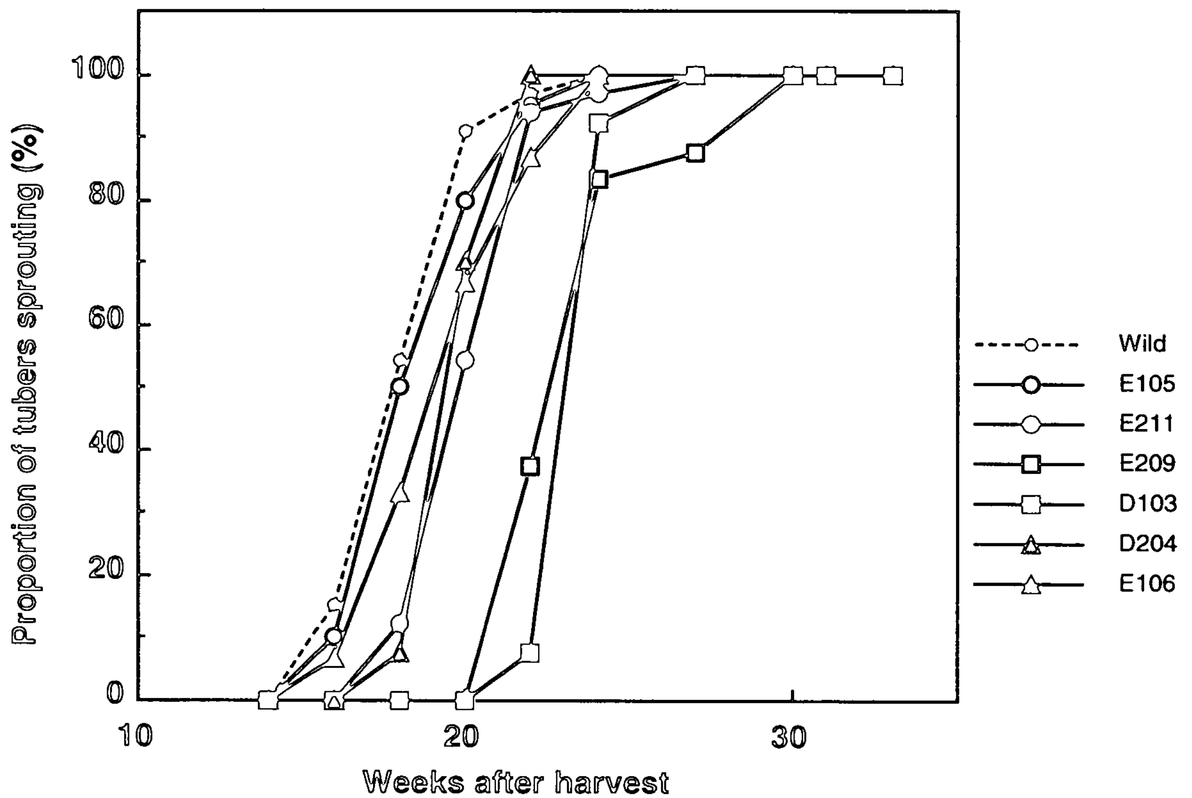


Figure 6.12 Effect of decreased D-enzyme activity on tuber sprouting
Harvested T1-2 tubers were kept at room temperature in the dark, and at indicated time points, the proportion of tubers producing sprouts (longer than 5 mm) was measured. Proportion of tubers sprouting means the proportion of number of sprouting tubers in total number of tubers.

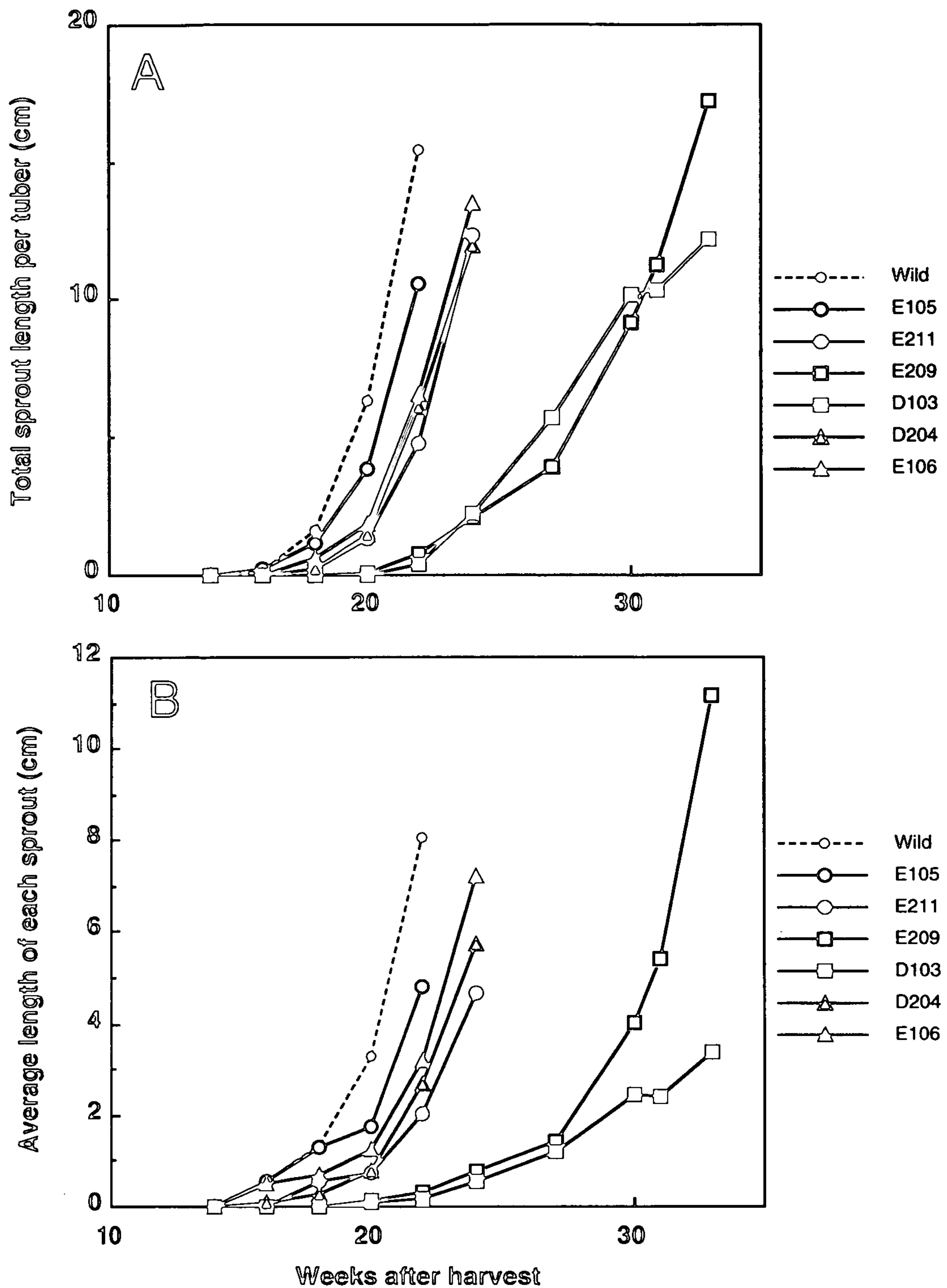


Figure 6.13 Effect of decreased D-enzyme activity on the growth of sprouts from seed tubers
Harvested T1-2 tubers were kept at room temperature in the dark as described in figure 6.12, and at indicated time points, the length of each sprout was measured. Total sprout length per tuber (A) and the average length of each sprout (B) are displayed.

6.4 Effect of reduced D-enzyme activity on starch in tubers

6.4.1 Amount of starch in tubers

The amount of starch in T1-2 tubers from wild type and three transgenic plants (E105, E211 and E209) were measured by the method described in section 2.4.9.1. However, there are no significant difference between wild and transgenic plants (table 6.3).

6.4.2 Structural analysis of starch

Starch granules were isolated from T1-2 tubers from wild type and three transgenic plants (E105, E211 and E209) by the method described in section 2.4.9.2. One g of starch granules from each plant was dispersed into 90 % (v/v) dimethyl sulphoxide (DMSO) to make 1 % (w/v) starch solution. The amylose content was analysed by the method described in section 2.4.9.4. However, no difference was found between wild type and transgenic plants (table 6.3).

In order to investigate the branching pattern of amylopectin molecules, starch from each plant was debranched completely by isoamylase, as described in section 2.4.9.5, and the chain length distribution was analysed by gel-filtration chromatography (Figure 6.14) and by HPAEC (Figure 6.15). However no significant difference was found between wild type and transgenic plants.

6.5 Discussion

Introduction of antisense genes successfully produced transgenic plants with decreased D-enzyme activity and two of them had less than 2 % of wild type D-enzyme activity. On the other hand introduction of sense genes did not affect D-enzyme activity significantly except two transformants which lost D-enzyme activity almost completely. The fact that the patatin promoter was less successful than the CaMV 35S promoter at reducing D-enzyme gene expression was unexpected, since the patatin promoter is expressed to a high level in tubers. D-enzyme activity in two transformants (E105 and E106) were variable (ranged from 2 % to 100 % of wild type) even in tubers within single plants. The reason for this variability and heterogeneity is not known, but similar results have been reported for several different genes, in various organs (van der Krol *et al.*, 1988; Kuipers *et al.*, 1994). However, the other four transgenic plants showed extremely low D-enzyme activity in all tubers

Table 6.3 Starch and amylose content in tubers of wild and transgenic tubers

Transformant	D-enzyme activity (%)	Starch content (μg / mg-fresh weight)	Amylose content (%)
Wild	100	73.8±11.8	19.1±1.2
E105	4.4	73.8±5.6	20.7±0.9
E211	1.4	85.9±9.3	19.9±0.8
E209	1.3	66.1±6.5	19.1±0.9

Assays were carried out on at least three independent plants.
The values for starch content and amylose content are the mean ± standard error.

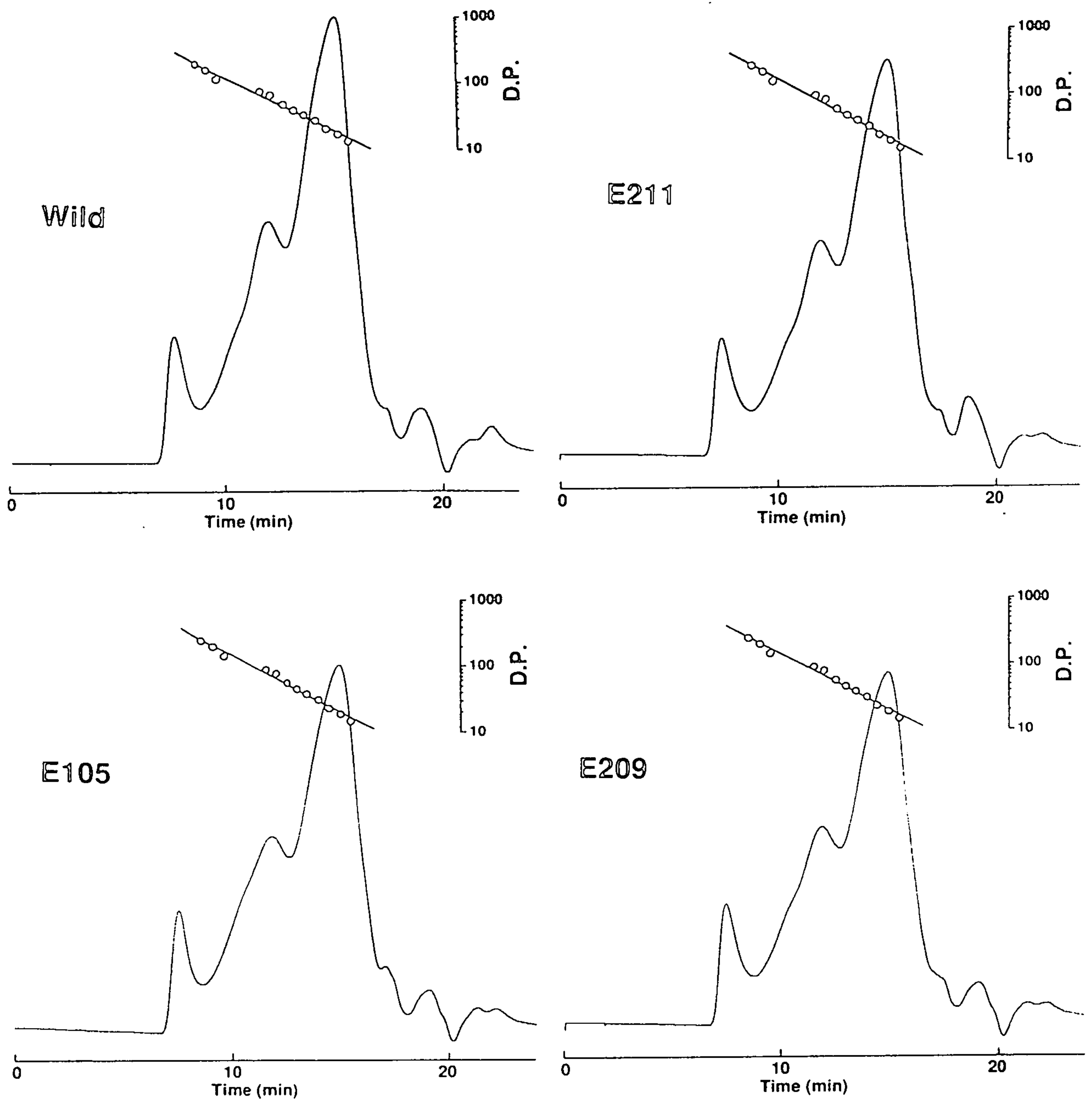


Figure 6.14 Analysis of debranched starch by gel-filtration chromatography
Starch granules were prepared from each tubers and solubilised in 90 % (v/v) DMSO, then completely digested with debranching enzymes as described in section 2.4.9.5. Debranched glucans were loaded onto gel-filtration columns (Sepharose 12 (10 x 300 mm, Pharmacia) linked with Superdex 30 (10 x 300 mm, Pharmacia)) with flow rate of 1 ml per min. Carbohydrates were detected with RI detector. The eluent was fractionated and the DP of debranched glucan in each fraction was calculated by measuring the total carbohydrate and reducing ends in each fraction.

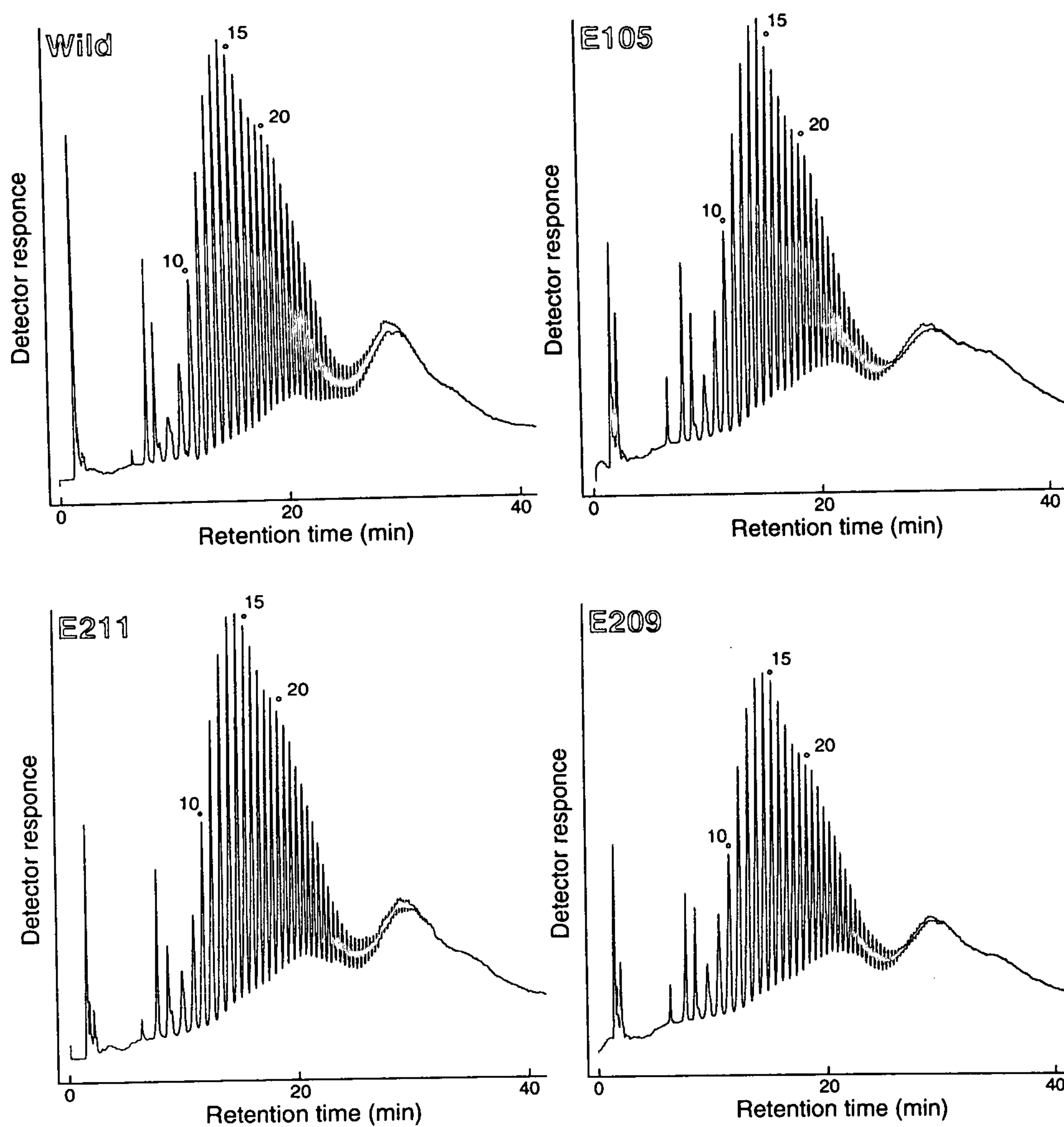


Figure 6.15 Analysis of debranched starch by high performance anion exchange chromatography (HPAEC)
Debranched glucans prepared in Figure 6.14 were analysed by HPAEC with pulsed amperometric detector . Conditions for HPAEC are described in section 2.4.5.

tested and seemed to be very stable even in the second generation produced from seed tubers (table 6.1).

Various phenotypic changes were observed in transgenic plants, however they were most evident in D103 and E209, the plants with the second and third lowest D-enzyme activity, respectively. These plants (E209 and D103) grew slower than wild type (Figure 6.7 and 6.9A), produced less leaves (Figure 6.9B), less mass of tubers (table 6.2) and the apical meristems suffered necrosis(Figure 6.8), whether the growth was started from *in vitro* shoots or seed tubers. Furthermore, tubers from these plants sprouted slower than wild type (Figure 6.12) and the growth of sprouts was also slower than wild type (Figure 6.13). These phenotypic changes also seemed to be found in another transformant, E211, the fourth lowest activity, but were not so evident in E105 (5th lowest), E106(6th lowest) and D204 (lowest). Thus these phenotypic changes correlated well with their D-enzyme activity, so may indicate the involvement of D-enzyme in these characteristics. However, in transformant D204, which showed the lowest D-enzyme activity, little difference was observed in the growth, tuber development and tuber sprouting. So the correlation of D-enzyme activity with these observations is not maintained, if D204 is included, and the involvement of D-enzyme in these characters is questionable. However, it may be important to consider the fact that D204 carries not the antisense but the sense copy of D-enzyme cDNA, thus the reduced D-enzyme activity (protein, and mRNA) is not caused by antisense RNA driven by the CaMV 35S promoter. The suppression of gene expression by introducing a sense transgene, called co-suppression, has been reported for several different genes, in various organs and cell types (van der Krol *et al.*, 1990; Hart *et al.*, 1992; Temple *et al.*, 1993), however the mechanism is not yet understood. Several models have been postulated , including the involvement of antisense RNA which is produced by an endogenous promoter (Grierson *et al.*, 1991; Mol *et al.*, 1991), transcriptional inactivation by DNA modifications (Napoli *et al.*, 1990), and a biochemical switch mechanism (Neuhaus *et al.*, 1991; Hart *et al.*, 1992). Whichever mechanism was used in the D204 transformant, it could be considered that there is a difference in the level of D-enzyme gene suppression in different organs, tissues or cells between D204 and antisense plants (E211, E209, E105 and E106).

In spite of the phenotypic change observed in transgenic plants with reduced D-enzyme activity, no significant difference was found in starch produced in tubers, either in its quantity and quality. Possible explanations for these results are as follows. First, D-enzyme is solely involved in starch degradation and does not play a role in starch synthesis in tubers. Second, the level of D-enzyme activity in wild type

tubers is in excess, so the decreased level of D-enzyme activity is still enough to catalyse normal starch metabolism. Third, the decreased D-enzyme activity does cause a change in starch structure, but the difference could not be found by the method employed here. Further discussion about the role of D-enzyme *in vivo* will be carried out in the following chapter.

Chapter 7

General discussion

7.1 Action of D-enzyme *in vitro*

From the extensive analysis of D-enzyme action on maltooligosaccharides, it has been believed that the maltose is preferred unit to be transferred from donor molecule to acceptor molecule. It was also found that when high molecular weight starch and glucose were used as donor and acceptor, respectively, maltotriose was the major product. The present work clearly demonstrates that D-enzyme does transfer much longer α-1,4-glucan chains or even α-1,4-glucan with highly branched structure (chapter 3). It is also demonstrated that high molecular weight amylose and amylopectin can serve as effective acceptors. These observations have not been made before, because the analysis of the transferred products has been done by either paper chromatography or TLC, where oligosaccharides with a DP of more than ten cannot be easily analysed. The employment of the new methods such as HPAEC or gel filtration led to the new discovery. However, even using these methods, it is not possible to determine, which molecule, maltooligosaccharide or high molecular weight starch, is preferred as donor or as acceptor by D-enzyme.

It was also newly demonstrated that D-enzyme catalyses an intra-molecular transglycosylation reaction on α-1,4-glucan to produce cyclic α-1,4-glucan. However we do not know which reaction, intra- or inter-molecular transglycosylation, mainly occurs *in vitro*. The current problem to answering this question is that there is no quantitative method to analyse the number of units transferred by the enzyme from high molecular weight starch to high molecular weight starch. Even qualitative analysis is very difficult unless the action occurs in the intra-molecular fashion, where easily detectable cyclic glucan can be produced. Establishment of new method seems to be necessary for further analysis of D-enzyme action.

It is of course very important to know whether this cyclisation reaction occurs *in vivo* to produce unidentified cyclic glucans. The non-reducing property of cyclic glucans is potentially very interesting since most of sugars in the cytosol or in the plastid occur as non-reducing forms such as sucrose, sugar-alcohols, or sugar phosphates. Furthermore, cyclic α-1,4-glucan is highly soluble in cold water (unlike linear amylose which is insoluble), which may also be an important property for starch metabolism *in vivo* .

7.2 Role of D-enzyme *in vivo* starch metabolism

It has been proposed that D-enzyme is involved in starch breakdown, to convert small oligosaccharides, which are no longer attacked by plastidic starch phosphorylase, into longer maltooligosaccharides, which can be attacked by phosphorylase. This view probably came from the following evidence. First, starch breakdown, in potato tuber and in leaf tissues, is believed to occur mainly by the phosphorolytic pathway rather than hydrolytic pathway (Stitt and ap Rees, 1980). Second, phosphorolytic breakdown of linear α-1,4-glucan stops when it is broken down to maltotetraose, because maltopentaose is the smallest substrate for this enzyme (Steup and Schächtele, 1981). Thus another system should be necessary for the complete breakdown of α-1,4-glucan. Third, in *E. coli*, amylomaltase, the equivalent enzyme in microorganisms, plays a role in converting maltotetraose into longer maltooligosaccharides which are further attacked by phosphorylase (Schwartz, 1987).

However, it is not known to what extent starch breakdown is performed by phosphorylase. Even if phosphorylase contributes to the breakdown of major proportions of starch, and the contribution of hydrolytic activity is small, a small amount of hydrolytic activity might be enough to degrade maltotetraose into glucose to achieve the complete utilisation of starch. It is also not known to what extent the bacterial system for exogenous maltooligosaccharide utilisation can be applied to plant starch breakdown which occurs inside the plastid. Furthermore, it is still questionable if plastidic starch phosphorylase is solely involved in starch breakdown. Other roles for this enzyme in the starch synthetic process may be possible. Both of the results in Chapter 5 and in St-Pierre and Brisson (1995), suggested that plastidic starch phosphorylase gene expression increased during starch synthesis. Thus the proposed role for D-enzyme is based largely on speculations and supported by no experimental evidence. The role of D-enzyme should be reconsidered from a wider point of view.

The knowledge obtained in Chapter 4 about the *in vitro* action of D-enzyme, greatly contributes to expand the possible roles of D-enzyme in starch metabolism *in vivo*. With the available information, including the results from Chapter 4, it is possible to propose at least five different functions for D-enzyme in starch metabolism either in starch synthesis or breakdown. First, it may be involved in starch breakdown to convert small oligosaccharides into larger molecules upon which starch phosphorylase can act. Second, it may be involved in starch breakdown, to generate from amylose and amylopectin, small cyclic glucans which can serve as substrates for

hydrolytic or phosphorolytic enzymes. It is generally considered that hydrolytic breakdown and phosphorolytic breakdown are the only two ways of starch breakdown, but it may now be very important to think about the transglycosidic breakdown of starch to produce soluble cyclic glucans. Third, it may be involved in the construction of amylopectin by transferring maltooligosaccharides from one side chain to another with its disproportionating activity. Fourth, it may be involved in the construction of the cluster structure of the amylopectin molecule, by transferring cluster units from one site to another site of the amylopectin molecule or from a soluble pre-amylopectin intermediate to the amylopectin molecule elongating along the surface of starch granule. Finally, during starch synthesis, D-enzyme and plastidic starch phosphorylase may be involved in the conversion of maltooligosaccharides produced by the 'trimming' activity of debranching enzyme on the elongating amylopectin molecule (Ball *et al.*, 1996), into glucose-1-phosphate which can be used again by AGPase for starch synthesis. This maltooligosaccharide recycling system during starch synthesis may explain the induction of D-enzyme and plastidic starch phosphorylase genes during starch synthesis (St-Pierre and Brisson, 1995, and section 5.2), and the substrate preference of plastidic starch phosphorylase for maltooligosaccharides rather than high molecular weight branched glucan.

Additionally, although it may be very speculative, cyclic glucan may be used as a mobile form of starch and transferred from plastids in source tissue to the plastids in sink tissue. It is undoubtedly true that most of the photoassimilated sugars which are not used for starch synthesis inside the chroloplast are transferred as sucrose to other plant tissues. Furthermore it is believed that starch accumulated inside the plastid in source organs is transformed into sucrose, by the action of many enzymes both in the stroma and cytosol, prior to phloem loading, then transferred to the sink organ. However, some starch may be mobilised as cyclic glucan, because this pathway requires less energy than the pathways using sucrose as the transported sugar. Furthermore this hypothesis may explain the existence of amylases and phosphorylases in the cytosol fraction (Beck and Ziegler, 1989), and the phloem specific localisation of β-amylase found in *Arabidopsis* plants (Wang *et al.*, 1995). In bacteria, a novel exogenous cyclic α-1,4-glucan utilisation system, which consists of cyclic α-1,4-glucan forming enzyme, cyclic α-1,4-glucan specific transport system and cyclic α-1,4-glucan hydrolases has recently been reported (Fiedler *et al.*, 1996). Thus there might be a similar cyclic glucan specific transporter system on the plastid membrane in plants.

The results obtained in Chapter 5 may support a function for D-enzyme in starch synthesis. D-enzyme mRNA accumulates under circumstances when starch biosynthesis is most active, but declines in amount under conditions when starch is broken down. D-enzyme gene expression seems to be co-ordinately regulated with other starch synthetic enzymes at the transcriptional level. However, the analysis of gene expression at the protein level suggests that the amount of RNA does not always correlate with the amount of protein. The abundant mRNA found in mature tuber greatly decreased during subsequent storage and sprouting stages, however the amount of protein did not change. In addition, the amount of mRNA in leaves increased dramatically over 24 h in response to added sucrose, but the amount of protein seemed to be constant. From all these results, it is also possible to suggest that D-enzyme could be involved in starch breakdown.

The transgenic potato plants with reduced D-enzyme activity produced normal tubers containing normal starch, but tubers from these plants sprouted slower than wild type (Figure 6.12) and the growth of sprouts was also slower than wild type (Figure 6.13, 14). Furthermore, these plants grew slower than wild type (Figure 6.8), produced less leaves (Figure 6.9) and necrotic apical shoots (Figure 6.7), particularly under restricted light conditions. All these results may suggest a function for D-enzyme in starch degradation or carbohydrate mobilisation.

Although this project aimed to investigate the roles for D-enzyme in starch metabolism, a final conclusion could not be reached. However, the accumulation of knowledge about the action of D-enzyme and about D-enzyme gene expression, as well as several biological and biochemical experimental materials, including transgenic plants, should be useful for future work.

7.3 Future work

7.3.1 Action of D-enzyme *in vitro*

D-enzyme activities in which transfer of a maltooligosaccharide unit from one side chain of amylopectin to another, and transfer of multi-branched cluster unit from one site of amylopectin to another molecule are proposed, but have not been demonstrated in this project. The former may be demonstrated by incubating D-enzyme with radio labelled amylopectin with low molecular mass in the presence and absence of amylopectin with high molecular mass as acceptor. D-enzyme should produce radioactive cyclic glucan from side chains of small amylopectin in the absence of acceptor molecule. However, if amylopectin is added as acceptor, D-enzyme may

produce radioactive large amylopectin. Large amylopectin, small amylopectin and cyclic glucan can be separated by the gel-filtration chromatography employed in Chapter 4.

7.3.2 Presence of cyclic glucan *in vivo*

It is of course very important to know whether the cyclisation reaction of D-enzyme occurs *in vivo* to produce cyclic glucans. For this purpose, one of the most important experiments for future is to investigate the presence of cyclic glucans in potato plants or in other plants. Although the presence of cyclic glucan in plant tissue has not been reported so far, this could be because nobody has looked for such molecules. Most starch analysed so far has been prepared from starch granules and not so much attention has been paid to the soluble glucans. Although the yield of such soluble glucan may be very low, it may provide interesting information not only about cyclic glucan but also other novel information about starch metabolism. The comparison of the transgenic potato plants with decreased D-enzyme activity produced in Chapter 6 with wild type should also be very useful for such experiments.

7.3.3 Regulation of D-enzyme gene expression

The regulation of synthesis of enzymes of starch metabolism has received much attention in recent years as genes encoding different enzymes have been isolated. The regulation of α-amylase synthesis in cereal grains by gibberellic acid is well documented (Akazawa and Hara-Nishimura, 1985). The expression of many other genes has been shown to be developmentally regulated, for example during seed or tuber formation (Koßmann *et al.*, 1991; Dry *et al.*, 1992). There is now growing evidence that developmentally regulated synthesis of many of these enzymes is controlled in large part by sucrose. An increasing number of genes encoding enzymes of starch metabolism has been shown to respond to sucrose, resulting in an increase in amounts of mRNA and enzyme. In potato these include ADP-glucose pyrophosphorylase S subunit (Müller-Röber *et al.*, 1990), granule-bound starch synthase (Visser *et al.*, 1989) and starch branching enzyme (Koßmann *et al.*, 1991). Numerous other genes are known to be activated by sucrose, including patatin (Rocha-Sosa *et al.*, 1989) and potato proteinase inhibitor II (Kim *et al.*, 1991). While the importance of sucrose in controlling enzyme synthesis is now established, much still needs to be learnt about the activation of gene expression by sucrose, and the control of enzyme activity in starch metabolism.

The transgenic potato plants transformed with a chimeric gene (the D-enzyme gene promoter sequence translationally fused to the GUS reporter gene) produced in Chapter 5 will be useful for further investigation of the regulation of D-enzyme gene expression. Preliminary analysis of GUS activity in several organs indicated that the 3.0 kb promoter sequence employed seems to contain the necessary genetic information for developmental regulation of D-enzyme gene expression. The analysis of the response of GUS gene expression to sucrose at the RNA and protein levels will answer the question whether the promoter sequence also contains genetic information for transcriptional control by metabolites (sucrose) and posttranscriptional control.

7.3.4 Analysis of transgenic potato plants

Further careful analysis of transgenic potato plants with decreased D-enzyme activity should be carried out. The analysis of starch was only carried out on starch prepared from starch granules in mature tubers in this project. However, it may be more interesting if the starch from different stages of tuber development, from leaves in different circumstances, and from different fractions (e.g.. soluble fraction) is analysed. It is also important to investigate whether D-enzyme is involved in starch synthesis or breakdown. For this purpose, the measurement of the rate of starch synthesis and starch breakdown in leaves or in isolated chloroplasts of transgenic plants and wild type may be informative. The phenotypic changes observed in transgenic plants are particularly interesting, and should be repeated by using statistically meaningful numbers of plants in well regulated growth conditions. From this point of view, potato may not be a good experimental plant because of its size and slow growth. Production of *Arabidopsis* plants with decreased D-enzyme activity by introducing an antisense gene, or isolation of D-enzyme deficient *Arabidopsis* mutants may represent a significant step towards understanding D-enzme function.

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Appendix

List of published papers

(1) Takaha, T., Yanase, M., Okada, S. and Smith, S. M. (1993) Disproportionating Enzyme (4-α-Glucanotransferase; EC 2.4.1.25) of Potato. *J. Biol. Chem.* 268, 1391-1396.

(2) Takaha, T., Yanase, M., Takata, H., Okada, S. and Smith, S. M. (1996) Potato D-enzyme catalyzes the cyclisation of amylose to produce cycloamylose, a novel cyclic glucan. *J. Biol. Chem.* 271, 2902-2908.

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Disproportionating Enzyme (4-α-Glucanotransferase; EC 2.4.1.25) of Potato

PURIFICATION, MOLECULAR CLONING, AND POTENTIAL ROLE IN STARCH METABOLISM*

(Received for publication, July 29, 1992)

Takeshi Takaha‡§, Michiyo Yanase§, Shigetaka Okada§, and Steven M. Smith‡¶

From the ‡Institute of Cell and Molecular Biology, University of Edinburgh EH9 3JH, United Kingdom and §Biochemical Research Laboratories, Ezaki Glico Company, Ltd., Osaka, Japan 555

Disproportionating enzyme (D-enzyme, 4-α-glucanotransferase; EC 2.4.1.25) has been purified to homogeneity from potato tubers and its activity characterized. The enzyme catalyzes the transfer of maltooligosaccharides from one 1,4-α-D-glucan molecule to another, or to glucose. Maltooligosaccharides are effective donor molecules, but short chain amylose and amylopectin may also function as donors. Enzyme activity is not affected by inorganic phosphate, 3-phosphoglycerate, or hexose phosphates. A cDNA clone encoding the enzyme was isolated using oligonucleotide probes derived from partial peptide sequences of the purified enzyme. The identity of the cDNA clone was confirmed by expression in *Escherichia coli* resulting in D-enzyme activity. The amino acid sequence deduced from the cDNA shows significant homology with a 4-α-glucanotransferase from *Streptococcus*. The deduced sequence indicates the presence of an amino-terminal plastid transit peptide of 52 amino acid residues and a mature polypeptide of 524 residues. D-enzyme mRNA is present in leaves, stems, roots, and stolons but is most abundant in developing and mature tubers. The amount of mRNA in leaves increases in response to light and to sucrose added to the medium. These results are discussed in terms of the function of D-enzyme in potato starch metabolism.

D-enzyme,[1] disproportionating enzyme, or 4-α-glucanotransferase (EC 2.4.1.25) was first found in potato tubers by Peat *et al.* (1956) but has since been found in many plant tissues (Lin and Preiss, 1988). It catalyzes glucan transfer from one 1,4-α-D-glucan molecule to another, or to glucose. Maltooligosaccharides are effective donors, although other donors may include soluble starch (Yoshio *et al.*, 1986) and amylopectin (Lin and Preiss, 1988). Maltooligosaccharides and glucose serve as acceptors. A maltosyl group is usually transferred except when maltotetraose is donor, when maltotriose is transferred. Despite this characterization of the activity of D-enzyme *in vitro*, its function in starch metabolism is unknown. It is generally believed that D-enzyme is involved in starch breakdown, to convert short oligosaccharides into longer chains upon which starch phosphorylase can act (Lee and Whelan, 1971; Lin and Preiss, 1988). Both enzymes are found in plastids, D-enzyme probably exclusively so (Lin *et al.*, 1988). A similar function has been proposed for *Escherichia coli*, in which D-enzyme (amylomaltase) and 1,4-α-D-glucan phosphorylase are encoded by genes of the *malA* operon (Schwartz, 1987).

The pathway of starch synthesis is relatively well understood, with three important enzymes having been characterized in detail (Preiss, 1991). ADP-glucose (ADP-Glc) pyrophosphorylase (EC 2.7.7.27) catalyzes ADP-Glc formation from glucose 1-phosphate and ATP. Starch synthases (EC 2.4.1.21) catalyze glucosyl transfer from ADP-Glc to the growing α-1,4-D-glucan chain, and starch branching enzyme (EC 2.4.1.18) catalyzes a glucanosyl transfer reaction creating α-1,6-linked branches. Starch synthesis is controlled in part by the regulation of enzyme synthesis but is also thought to be regulated to a significant extent by substrates and effectors of ADP-Glc pyrophosphorylase (Preiss, 1991). These regulators include inorganic phosphate, 3-phosphoglyceric acid, glucose 1-phosphate, fructose 6-phosphate, and ATP.

The control of starch breakdown is less clearly understood and may differ depending upon whether reserve or assimilatory starch is involved. The breakdown of reserve starch in cereal seeds is well documented, involving the controlled synthesis and secretion of α-amylase (EC 3.2.1.1) which hydrolyzes starch to glucose (Beck and Ziegler, 1989). This hydrolytic breakdown occurs in the endosperm tissue which lacks an intact cell structure. In contrast, the mobilization of plastidic starch, such as in leaves or potato tubers, probably involves phosphorolytic breakdown to glucose 1-phosphate by starch phosphorylase (EC 2.4.1.1). Starch breakdown in plastids may be controlled in part by the synthesis of relevant enzymes; but in contrast to starch synthesis, the regulation of enzyme activity by effector molecules has not been reported, except that high concentrations of phosphate will promote phosphorolysis through mass action (Steup *et al.*, 1976).

The regulation of synthesis of enzymes of starch metabolism has received much attention in recent years as genes encoding different enzymes have been isolated. The regulation of α-amylase synthesis in cereal grains by gibberellic acid is well documented (Akazawa and Hara-Nishimura, 1985). The expression of many other genes has been shown to be developmentally regulated, for example during seed or tuber formation (Kossmann *et al.*, 1991; Day *et al.*, 1992). There is now

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X68664.

¶ To whom correspondence should be addressed: Institute of Cell and Molecular Biology, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, United Kingdom. Tel.: 031-650-5318; Fax: 031-650-5392.

[1] The abbreviations used are: D-enzyme, disproportionating enzyme; IPTG, isopropyl 1-thio-β-D-galactopyranoside; HPLC, high performance liquid chromatography.

growing evidence that developmentally regulated synthesis of many of these enzymes is controlled in large part by sucrose. An increasing number of genes encoding enzymes of starch metabolism have been shown to respond to sucrose, resulting in an increase in the amounts of mRNA and enzyme. In potato these include ADP-Glc pyrophosphorylase S subunit (Müller-Röber *et al.*, 1990), granule-bound starch synthase (Visser *et al.*, 1989), and starch branching enzyme (Kossmann *et al.*, 1991). An activation of gene transcription by sucrose is implied from studies with the *GUS* reporter gene (Visser *et al.*, 1991). Numerous other genes are known to be activated by sucrose, including patatin (Rocha-Sosa *et al.*, 1989) and potato proteinase inhibitor II (Kim *et al.*, 1991). Although the importance of sucrose in controlling enzyme synthesis is now established, much still needs to be learned about the activation of gene expression by sucrose and the control of enzyme activity in starch metabolism.

Here we report the purification of D-enzyme from potato tubers and show that its activity is not regulated by effector molecules known to regulate starch metabolism. Isolation of a cDNA clone has enabled us to show that D-enzyme gene expression is developmentally regulated in potato and is controlled by light and by sucrose in potato leaves.

EXPERIMENTAL PROCEDURES

Plant Material—*Solanum tuberosum* L. (cv. May Queen) was obtained either from a local potato merchant in Osaka or from the Scottish Office Agriculture and Fisheries Department, Edinburgh. Plants were grown in compost in a glasshouse. For induction of D-enzyme gene expression, detached leaves were incubated in Murashige and Skoog (MS) medium (Flow Laboratories, Irvine, CA).

Assay of D-enzyme—D-enzyme activity was assayed in 100 μl of reaction mixture containing 100 mM Tris-Cl buffer (pH 7.0), 5 mM 2-mercaptoethanol, 1% (w/v) maltotriose, and enzyme. The reaction mixture was incubated at 37 °C for 10 min and terminated by immersing the reaction tubes in boiling water for 3 min. Released glucose was measured by the glucose oxidase method (Barham and Trinder, 1972). One unit of activity is defined as the amount of enzyme which produces 1 μmol of glucose/min under these assay conditions.

Purification of Potato D-enzyme—Potato tubers (2.5 kg) were washed, peeled, and homogenized in the presence of 20 mM Tris-Cl (pH 7.5) and 5 mM 2-mercaptoethanol. After centrifugation and filtration through a 0.45-μm membrane, the homogenate was applied to a Q-Sepharose fast flow column (16 × 100 mm, Pharmacia LKB Biotechnology Inc.) and washed with 150 mM NaCl in 20 mM Tris-Cl (pH 7.5) containing 5 mM 2-mercaptoethanol (buffer A). D-enzyme was eluted with buffer A containing 450 mM NaCl and dialyzed against buffer A. Concentrated Tris buffer, ammonium sulfate, and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate, and 5 mM 2-mercaptoethanol. The solution was applied to a phenyl-Toyopearl 650M (Tosoh, Japan) column (10 × 100 mm) and eluted with a linear gradient of 500–0 mM ammonium sulfate in buffer A. Active fractions were pooled and dialyzed against buffer A. The dialysate was concentrated by an Amicon Centricon 30 microconcentrator and applied to a PL-SAX HPLC column (Polymer Laboratories, U. K.). The enzyme was eluted with a linear gradient of 150–400 mM NaCl in buffer A. Active fractions were pooled and concentrated by Centricon 30 microconcentrator.

Thin Layer Chromatography—20 μl of reaction mixtures were spotted on Silica Gel 60 thin layer plates (Merck) and developed three times in 1-butanol/ethanol/water (5:5:3). After chromatography, compounds were detected by spraying 6 M sulfuric acid in methanol and baking at 140 °C for 3–10 min.

SDS-Polyacrylamide Gel Electrophoresis and Measurement of Molecular Weight—The relative molecular mass was measured by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970), using prestained SDS-polyacrylamide gel electrophoresis standards (Bio-Rad) as molecular weight markers.

Amino Acid Sequence Analysis—Purified D-enzyme was applied to reverse-phase HPLC using a C4 column (250 × 4.6 mm; YMC Biochemicals, Japan) and eluted with a linear 0–40% (v/v) gradient of acetonitrile with 0.1% (v/v) trifluoroacetic acid and freeze-dried. 500 pmol of the protein was used for amino-terminal amino acid sequencing using an automated peptide sequenator (Shimazu, Japan). 2 nmol of the protein was digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma) in the presence of 12 M urea and 100 mM Tris-Cl (pH 8.0). The fragments generated were separated by reverse-phase HPLC using C4 and C18 columns (250 × 4.6 mm; YMC Biochemicals) with a gradient of acetonitrile containing 0.1% (v/v) trifluoroacetic acid. Two fragments that were well separated from others were collected and lyophilized and then used for amino acid sequencing.

Construction and Screening of Potato cDNA Library—cDNA was synthesized from poly(A)$^+$ RNA prepared from immature potato tubers (1 cm) using the cDNA synthesis kit from Pharmacia. This cDNA was ligated into λgt10 arms via *Eco*RI/*Not*I adapters and packaged using Gigapack II gold packaging extracts (Stratagene). The cDNA library was plated on *E. coli* C600*Hfl* cells and transferred to Hybond N$^+$ membrane filters (Amersham Corp.). Oligonucleotide hybridization was done in 6 × SSC containing 5 × Denhardt's solution, 0.1% (w/v) SDS, 0.05 mg ml^{-1} denatured salmon sperm DNA, and synthetic oligonucleotide probe labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase at 35 °C overnight. The filters were washed twice in 6 × SSC containing 0.1% (w/v) SDS at 35 °C for 15 min and autoradiographed.

Subcloning and DNA Sequencing—Phage DNA was purified by the plate lysate method (Maniatis *et al.*, 1982), and the cDNA insert was digested with *Not*I and subcloned into the *Not*I site of pBluescript II SK+ (Stratagene) in both orientations. These plasmids were designated pDPE-102P (forward orientation relative to β-galactosidase) and pDPE-102N (reverse orientation). A series of deletion derivatives from both ends of the cDNA was obtained using the method of Henikoff (1984) and used for DNA sequencing of both strands. DNA sequencing was performed using the dideoxy chain termination method of Sanger *et al.* (1977). Comparison of the deduced amino acid sequence with other sequences was performed using the GAP program (Devereux *et al.*, 1984).

Expression of the D-enzyme Gene in E. coli—Plasmid pKK233-2 (Pharmacia) was used to construct a D-enzyme gene expression vector. A new *Nco*I site was introduced into the translational initiation sequence of pDPE-102P using an *in vitro* mutagenesis system (U. S. Biochemical Corp.) and a synthetic 21-mer mutagenic primer (5′-GCGCTATCCATGGTAATGAAT-3′). The resulting plasmid pDPE-102P(Nco) was digested completely with *Pst*I and partially with *Nco*I, and a 2.1-kilobase *Nco*I/*Pst*I fragment which covers the entire coding sequence was purified and subcloned into *Nco*I and *Pst*I sites of pKK233-2. For the purification, *E. coli* cells carrying this plasmid were cultured with the inducer IPTG (2 mM) for 6 h at 15 °C then harvested by centrifugation and disrupted by sonication in buffer A at 4 °C. This crude extract was centrifuged, and the supernatant was filtered through a 0.45-μm membrane and then applied to a Q-Sepharose fast flow column. Further purification steps are the same as described above.

Isolation and Analysis of RNA—Total RNA was extracted from frozen plant materials by the following methods. About 1 g of frozen tissue was ground in a mortar and pestle under liquid nitrogen and transferred to a centrifuge tube. 4 ml of extraction buffer (100 mM Tris-Cl (pH 8.5), 10% (w/v) triisopropylnaphthalenesulfonic acid (Kodak), and 6% (w/v) 4-aminosalicylic acid) and 4 ml of phenol:chloroform (1:1 (v/v)) were added to the tube, voltexed, and then centrifuged at 3,500 rpm for 20 min. The upper aqueous phase was transferred to a new centrifuge tube and precipitated with 0.10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. The precipitate was collected by centrifugation and dissolved in 1 ml of diethyl pyrocarbonate-treated water. RNA was selectively precipitated by the addition of an equal volume of 5 M LiCl and incubation at 4 °C overnight. The RNA precipitated was collected by centrifugation, washed with 70% (v/v) ethanol, and dried. Poly(A)$^+$ RNA was isolated using an oligo(dT)-cellulose column (Maniatis *et al.*, 1982). RNA was electrophoresed on formaldehyde-agarose gels (Maniatis *et al.*, 1982) and blotted onto Hybond N membrane filters (Amersham Corp.). The filters were hybridized with a ^{32}P-labeled 1.0-kilobase *Eco*RI fragment of pDPE-102P in buffer containing 6 × SSPE, 5 × Denhardt's solution, 40% (v/v) formamide, 0.1% (w/v) SDS, and 0.05 mg ml^{-1} denatured salmon sperm DNA at 42 °C for 18 h. ^{32}P-Labeled DNA probes were prepared using a random oligonucleotide primer method (Feinberg and Vogelstein, 1983). The filters were washed twice in 1 × SSPE containing 0.1% (w/v) SDS at 42 °C for 15 min and autoradiographed.

RESULTS

Purification and Properties of D-enzyme—The enzyme was isolated from mature potato tubers and assayed by its ability to release glucose from maltotriose. Purification employed the four chromatography procedures shown in Table I. These steps consisted of anion exchange fast protein liquid chromatography using Q-Sepharose, hydrophobic fast protein liquid chromatography using phenyl-Toyopearl, and two cycles of anion exchange HPLC using PL-SAX. The purified enzyme shows a single band on SDS-polyacrylamide gel electrophoresis, with an estimated molecular weight of 60,000 (Fig. 1, *lane 2*). The increase in specific activity of the enzyme during purification (Table I) is an underestimate since hydrolases contaminate the preparation at early stages of the purification.

To confirm the identity of D-enzyme and to investigate its substrate specificity further, the activity of the enzyme on a range of maltooligosaccharides was investigated. Substrates were incubated with or without enzyme for 18 h then separated by TLC. Fig. 2*A* shows that no transglycosylation reaction could be detected with maltose as substrate, but that maltotriose, maltotetraose, and maltopentaose were effective substrates, producing other maltooligosaccharides and glucose, but not maltose. Note that when maltopentaose was substrate, high molecular weight oligosaccharides were produced which remained at the origin during TLC. To investigate the proposal that D-enzyme can transfer maltooligosyl groups from starch to glucose (Lin and Preiss, 1988; Yoshio *et al.*, 1986), short chain amylose and amylopectin were incubated with enzyme for 4 h in the presence and absence of glucose (Fig. 2*B*). Small oligosaccharides without maltose were produced from both starch substrates only in the presence of glucose. The results show that D-enzyme catalyzes the transfer of maltooligosaccharides from starch to glucose. Hexose phosphates could not substitute for glucose in this reaction (results not shown). Numerous molecules known to regulate starch metabolism (for example by allosteric regulation of ADP-Glc pyrophosphorylase) were tested for their ability to affect D-enzyme activity. 3-Phosphoglycerate, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, and inorganic phosphate were each tested at 10 mM and found to have no effect on the production of glucose from maltotriose by D-enzyme (results not shown).

TABLE I
Purification of D-enzyme from 2.5-kg tubers

Step	Total activity[a]	Total protein[b]	Specific activity
	units	*mg*	*units/mg*
Crude extract	6,202.2	4,168.8	1.49
Q-Sepharose	474.7	180.0	2.64
Phenyl-Toyopearl	48.6	6.27	7.75
1st PL-SAX (HPLC)	23.7	0.67	35.7
2nd PL-SAX (HPLC)	21.8	0.46	47.5

[a] One unit of activity was defined as the amount of enzyme which produced 1 μmol of glucose/min at 37 °C.
[b] Protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

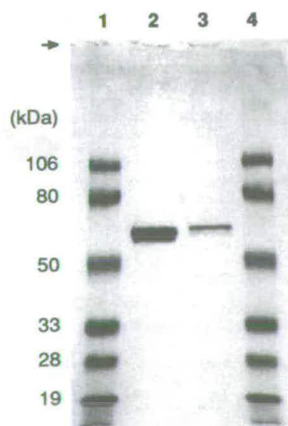

FIG. 1. **SDS-polyacrylamide gel electrophoresis of potato D-enzyme purified from tubers and recombinant *E. coli*.** D-enzyme was purified as described in Table I from potato tubers and recombinant *E. coli*, fractionated by SDS-polyacrylamide gel electrophoresis, and stained with Coomassie Blue. *Lanes 1* and *4*, marker proteins; *lane 2*, enzyme purified from tubers; *lane 3*, enzyme purified from *E. coli*. *Numbers* on the *left* are the estimated molecular masses of marker proteins, redetermined by the manufacturer (Bio-Rad). The origin is marked with an *arrow*.

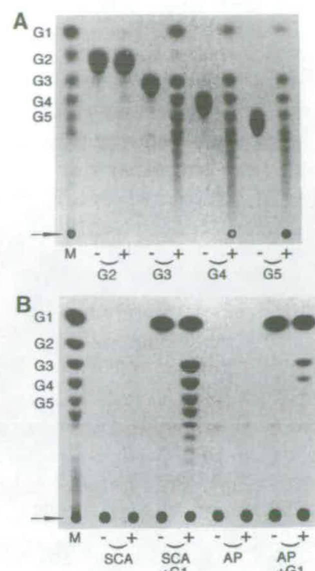

FIG. 2. **Thin layer chromatogram of reaction products from the activity of purified D-enzyme.** *Panel A*, action on oligosaccharides. The reaction mixture (30 μl) containing 300 μg of substrates was incubated with (+) or without (−) enzyme (0.1 units) at 37 °C for 18 h. 20 μl of the reaction mixture was analyzed by thin layer chromatography and stained with H_2SO_4 in methanol. *Panel B*, action on starch. The reaction mixture (30 μl) containing 300 μg of substrates was incubated with (+) or without (−) enzyme (0.01 units) at 37 °C for 4 h in the presence (*+G1*) or absence of glucose (40 mM). 20 μl of the reaction mixture was analyzed by thin layer chromatography and stained with H_2SO_4 in methanol. *M*, standard maltooligosaccharides; *G1*, *G2*, *G3*, *G4*, *G5*, *AP*, and *SCA* are glucose, maltose, maltotriose, maltotetraose, maltopentaose, amylopectin, and short chain amylose (degree of polymerization approximately 18, Amylose EX-I, Hayashibara Biochemical Laboratories, Japan), respectively. The origins are marked with *arrows*.

Isolation of a cDNA Clone Encoding D-enzyme—Amino-terminal amino acid sequencing of purified D-enzyme was carried out by automated Edman degradation, revealing the first 5 residues (AVPAV). To obtain information about internal amino acid sequences, purified D-enzyme was digested with trypsin and two peptides isolated by reverse-phase HPLC. Sequence analysis produced amino acid sequences for both peptides (HLAALEEVYQSEK and VNYSTISEIK). A mixed (32-fold redundant) oligonucleotide probe encoding EEVYQ was synthesized (5′-TGRTANACYTCYTC-3′) to use as a hybridization probe to screen a cDNA library. cDNA was synthesized from poly(A)$^+$ RNA isolated from immature (1-cm) tubers and a library constructed in bacteriophage λgt10 vector. Three positive plaques were isolated from 5×10^4 recombinant plaques, one of which was shown by partial

nucleotide sequence analysis to encode D-enzyme. This clone was used as a probe to screen a further 2×10^4 plaques, and three more clones were isolated. All four clones had cDNA inserts of about 2 kilobase pairs and encoded D-enzyme as shown by partial nucleotide sequencing. Only one cDNA contained a poly(A) tail, but otherwise no differences in nucleotide sequence were detected in the 3′ ends of each cDNA. One clone, pDPE-102, was selected for detailed analysis.

Nucleotide Sequence of D-enzyme cDNA—The sequence of pDPE-102 was determined completely and is shown in Fig. 3. The 5′ end consists of 13 nucleotides, followed by an open reading frame of 1728 nucleotides, a 3′-untranslated sequence of 368 nucleotides, but no poly(A) tail. The cDNA clone with the poly(A) tail had a 3′-untranslated region which was about 10 nucleotides longer than pDPE-102 (the poly(A) interfered with nucleotide sequencing). This observation suggests that the sequence 5′-AATAAA-3′ *underlined* in Fig. 3 (position 2072) could be a polyadenylation signal (Proudfoot and Brownlee, 1976). The sequence around the putative translation initiation site 5′-ATCAATGGC 3′ is similar to a proposed plant mRNA consensus, 5′-AACAATGGC-3′ (Lütcke *et al.*, 1987). The open reading frame encodes a polypeptide of 576 amino acid residues (M_r 64,950), but the amino acid sequence found at the amino terminus of the purified enzyme (*arrowed* in Fig. 3) starts at amino acid position 53. We deduce that the first 52 amino acid residues constitute a transit peptide for targeting to the plastid. This sequence is rich in basic and hydroxylated residues, similar to other transit peptides (Keegstra and Olsen, 1989). The remaining 524 residues constitute the mature enzyme (M_r 59,500). The two tryptic peptides found in the enzyme are *underlined* in Fig. 3, helping to confirm the identity of the cDNA clone. The identity is further confirmed by comparison of the deduced sequence of the potato enzyme with the equivalent enzyme (amylomaltase) from microorganisms. Potato D-enzyme shows significant homology (40% identity) with that of *Streptococcus pneumoniae* (Lacks *et al.*, 1982) but low homology (23% identity) with that of *E. coli* (Pugsley and Dubrevil, 1988).

Expression of Potato D-enzyme in E. coli—Expression of the cDNA clone in *E. coli* was sought to obtain final confirmation of its identity. The plasmid vector pKK233–2 containing a *trc* promoter (with 17 base pairs between the *trp* −35 region and the *lac* UV5 −10 region) was employed to direct expression of the complete cDNA open reading frame (see "Experimental Procedures"). Following induction of gene expression with IPTG at 37 °C, D-enzyme activity was measured in extracts of *E. coli* cells containing either the plasmid vector or recombinant plasmid, pKK233-DPE. No activity was detected in either set of cells. However, when cells were grown at reduced temperature following IPTG induction, significant D-enzyme activity was detected in extracts of cells

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   1 ATTCTCATTATCAATGGCAATTCACACTTGTTTCTCACTAATACCTTCTTCTTTCTCTTCTCCCAAATTGCCATACCCCAAAAACACTAC
                   M  A  I  H  T  C  F  S  L  I  P  S  S  F  S  S  P  K  L  P  Y  P  K  N  T  T
  91 TTTTCAATCTCCTATCCCAAAATTATCTAGACCCACTTTCATGTTTGATCGGAAAGGTTCCTTTCAAAATGGCACCGCTGCCGTTCCTGC
       F  Q  S  P  I  P  K  L  S  R  P  T  F  M  F  D  R  K  G  S  F  Q  N  G  T  A  A  V  P  A
 181 TGTAGGTGAGGATTTTCCGATTGATTATGCTGATTGGTTGCCAAAACGAGACCCAAACGATCGTAGAAGAGCTGGAATTTTGCTCCATCC
       V  G  E  D  F  P  I  D  Y  A  D  W  L  P  K  R  D  P  N  D  R  R  R  A  G  I  L  L  H  P
 271 GACGTCGTTTCCTGGACCTTATGGTATTGGTGACCTTGGCCCTCAGGCTTTTAAGTTCCTTGATTGGCTTCATCTTGCTGGTTGCTCCCT
       T  S  F  P  G  P  Y  G  I  G  D  L  G  P  Q  A  F  K  F  L  D  W  L  H  L  A  G  C  S  L
 361 TTGGCAGGTTCTTCCACTTGTACCGCCTGGAAAGAGAGGCAATGAAGATGGATCACCCTATTCAGGACAGGATGCAAATTGTGGAAACAC
       W  Q  V  L  P  L  V  P  P  G  K  R  G  N  E  D  G  S  P  Y  S  G  Q  D  A  N  C  G  N  T
 451 ACTTCTGATTTCTCTTGAAGAGCTTGTTGATGATGGTTTACTGAAGATGGAGGAGCTTCCGGAGCCACTACCTACAGATCGTGTCAATTA
       L  L  I  S  L  E  E  L  V  D  D  G  L  L  K  M  E  E  L  P  E  P  L  P  T  D  R  V  N  Y
 541 CTCGACTATATCTGAGATAAAAGATCCTTTAATAACCAAGGCAGCAAAGAGGCTTCTCTCCAGTGAAGGGGAACTGAAAGACCAGCTCGA
       S  T  I  S  E  I  K  D  P  L  I  T  K  A  A  K  R  L  L  S  S  E  G  E  L  K  D  Q  L  E
 631 GAACTTTCGCCGGGATCCAAATATTTCGAGTTGGCTGGAGGATGCTGCTTATTTTGCTGCCATAGACAACTCTGTAAACACTATTAGCTG
       N  F  R  R  D  P  N  I  S  S  W  L  E  D  A  A  Y  F  A  A  I  D  N  S  V  N  T  I  S  W
 721 GTATGATTGGCCTGAACCATTGAAAAATCGCCATCTTGCAGCTCTAGAAGAAGTTTATCAAAGTGAAAAGGATTTTATTGACATATTCAT
       Y  D  W  P  E  P  L  K  N  R  H  L  A  A  L  E  E  V  Y  Q  S  E  K  D  F  I  D  I  F  I
 811 TGCACAACAGTTCTTGTTCCAACGACAATGGAAAAAAGTTCGTGACTATGCACGATCCAAAGGAATCAGTATAATGGGAGACATGCCAAT
       A  Q  Q  F  L  F  Q  R  Q  W  K  K  V  R  D  Y  A  R  S  K  G  I  S  I  M  G  D  M  P  I
 901 ATATGTTGGATATCACAGTGCTGATGTTTGGGCCAACAAGAAACAATTTTTGCTGAATAGGAAAGGTTTCCCTCTTATAGTTAGTGGTGT
       Y  V  G  Y  H  S  A  D  V  W  A  N  K  K  Q  F  L  L  N  R  K  G  F  P  L  I  V  S  G  V
 991 TCCTCCAGACGCCTTTAGTGAAACTGGTCAACTATGGGGCAGCCCTCTCTATGATTGGAAAGCCATGGAGAAGGATGGATTTTCATGGTG
       P  P  D  A  F  S  E  T  G  Q  L  W  G  S  P  L  Y  D  W  K  A  M  E  K  D  G  F  S  W  W
1081 GGTACGCCGAATTCAACGTGCAACGGATCTTTTTGATGAATTTAGGATAGATCACTTTAGAGGATTTGCTGGATTTTGGGCTGTTCCTTC
       V  R  R  I  Q  R  A  T  D  L  F  D  E  F  R  I  D  H  F  R  G  F  A  G  F  W  A  V  P  S
1171 TGAGGAAAAAATTGCAATTCTGGGACGGTGGAAGGTGGGACCTGGAAAGCCTTTGTTTGATGCTATCTTACAAGCTGTTGGGAAGATCAA
       E  E  K  I  A  I  L  G  R  W  K  V  G  P  G  K  P  L  F  D  A  I  L  Q  A  V  G  K  I  N
1261 TATTATAGCAGAAGACTTGGGAGTAATTACCGAGGACGTTGTTCAGCTAAGAAAGTCCATTGAGGCACCTGGAATGGCTGTACTCCAGTT
       I  I  A  E  D  L  G  V  I  T  E  D  V  V  Q  L  R  K  S  I  E  A  P  G  M  A  V  L  Q  F
1351 TGCATTTGGCAGTGACGCTGAAAACCCTCATTTACCTCACAATCATGAGCAGAACCAAGTAGTGTATACTGGAACACATGACAATGATAC
       A  F  G  S  D  A  E  N  P  H  L  P  H  N  H  E  Q  N  Q  V  V  Y  T  G  T  H  D  N  D  T
1441 GATCCGAGGTTGGTGGGATACTTTGCCACAGGAAGAGAAATCCAATGTACTAAAGTATTTATCAAATATTGAGGAAGAGGAAATATCACG
       I  R  G  W  W  D  T  L  P  Q  E  E  K  S  N  V  L  K  Y  L  S  N  I  E  E  E  E  I  S  R
1531 GGGCTTGATCGAAGGTGCAGTTTCTTCTGTAGCCCGTATTGCAATTATACCGATGCAGGATGTTCTTGGGCTTGGGAGTGATTCCAGAAT
       G  L  I  E  G  A  V  S  S  V  A  R  I  A  I  I  P  M  Q  D  V  L  G  L  G  S  D  S  R  M
1621 GAACATTCCAGCAACTCAGTTTGGAAACTGGAGTTGGAGGATACCTAGTTCAACTAGCTTTGACAACCTGGATGCAGAAGCAAAAAAGCT
       N  I  P  A  T  Q  F  G  N  W  S  W  R  I  P  S  S  T  S  F  D  N  L  D  A  E  A  K  K  L
1711 AAGAGATATACTTGCAACTTATGGGCGGTTGTGATGATGAATGATGATTAAGATGTTATGTCCCCTGGGAATCAGGGGAGGACCTAGGCT
       R  D  I  L  A  T  Y  G  R  L
1801 GTAAGGACGTTCTCAGGCTTGGGACACAAATTGCTGAGAGCATAAACGATCTAAACAAGGTTTTCCTGCCACAGTTCACCTCATTGCTGC
1891 AAAGGAACGCCTGCATTTTCAATTTTCTGAACTTTAGGCTCACTGGGGAAGCAGACAAGGAAAGCTAAAACGCGTGCTTTGGATTCTATT
1981 ATTCTACATTAGCTGGTATAAACTACGAATGCTAGAGCCTTTCCATATTTATGCAGAAAGACTTCATAGTTTGAATGTCACCTGACCTAA
2071 AAATAAATAATGCATGTTTTTGTATTTACTATTTGGTCG
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FIG. 3. **Nucleotide and deduced amino acid sequences of D-enzyme cDNA.** The DNA sequence of pDPE-102 was determined completely on both strands. The amino-terminal end of the mature protein is marked by an *arrow*. Peptide sequences derived from the purified enzyme are *boxed*.

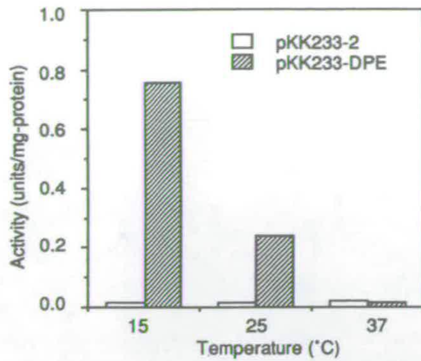

FIG. 4. **D-enzyme activity of the extract of recombinant *E. coli*.** *E. coli* cells transformed with pKK233-2 and pKK233-DPE were cultured with the inducer IPTG for 6 h at the indicated temperatures. Extracts prepared by sonication and centrifugation were used to assay for D-enzyme activity and protein.

containing the recombinant plasmid but not in controls (Fig. 4). Furthermore, the enzyme synthesized by cells at 15 °C was purified (using the methods described in Table I) and shown to have a molecular weight of approximately 65,000 (Fig. 1, *lane 3*). This molecular weight corresponds with that of the D-enzyme precursor (M_r 65,000) but not with that of endogenous amylomaltase of *E. coli* (M_r 78,360; Pugsley and Dubrevil, 1988). The successful synthesis of biologically active recombinant proteins in *E. coli* at reduced temperatures has been reported previously (Mori *et al.*, 1991), but we do not know its cause. However, these experiments successfully established the identity of the D-enzyme cDNA clone.

D-enzyme Gene Expression—To investigate the control of D-enzyme gene expression in potato plants, equal amounts of total RNA isolated from a range of organs were analyzed by gel electrophoresis, blotting, and hybridization with the D-enzyme cDNA probe. These results (Fig. 5*A* and longer autoradiograph exposures not shown) show that D-enzyme RNA is detected in all organs tested, including leaves, petioles, stems, roots, and stolons, but is most abundant in developing and mature tubers.

Leaves were next chosen as a simple experimental system to investigate factors which may regulate D-enzyme gene expression. In the first experiment, total RNA was isolated at 3-h intervals from leaves of plants growing in a 12-h light/dark cycle. Gel blot analysis (Fig. 5*B*) shows that D-enzyme RNA increases in amount during the light period and then declines in the dark. To investigate what may cause the increase in the light, leaves were removed after the dark period and then incubated on different sugars, either in the light or the dark for a further 24 h. RNA gel blot analysis of equal amounts of total RNA from each (Fig. 5*C*) shows that light alone causes a significant increase in the amount of D-enzyme RNA and that this is increased further by sucrose or glucose, but not by mannitol. Sucrose and glucose can also cause an increase in amount in the dark. Thus, light and metabolizable sugars appear to show an additive effect in stimulating D-enzyme RNA accumulation in potato leaves. The amount of RNA correlates well with the amount of starch revealed in each leaf by iodine staining (Fig. 5*D*).

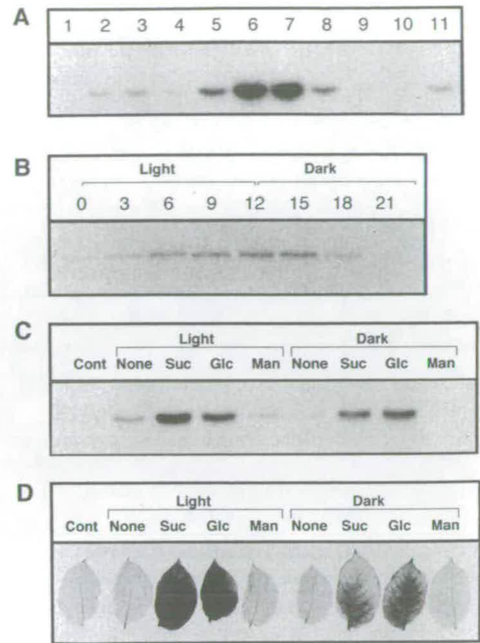

FIG. 5. **Northern blot analysis of D-enzyme gene expression.** *Panel A*, organ-specific expression pattern of D-enzyme gene. Total RNA (20 μg) from the following organs was loaded in each *lane* and probed with ^{32}P-labeled cDNA for D-enzyme. *Lane 1*, leaves; *lane 2*, petioles; *lane 3*, stems; *lane 4*, roots; *lane 5*, stolons; *lane 6*, developing tubers; *lane 7*, mature tubers; *lanes 8* and *9*, mature tubers stored in cold room (4 °C) for 2 and 6 months; *lane 10*, sprouting tubers; *lane 11*, dark-grown shoots. *Panel B*, induction of the expression of D-enzyme gene by light. Total RNA (15 μg) from leaves at 3-h intervals through the day (shown above) was loaded in each *lane* and probed with ^{32}P-labeled cDNA. *Panel C*, induction of the expression of D-enzyme gene in detached leaves by light and carbohydrates. Total RNA (15 μg) of each of the following samples was hybridized to a ^{32}P-labeled cDNA probe. *Cont*, leaves directly harvested at the end of the dark period; in all other *lanes* leaves were harvested after a further 24-h incubation in the light or dark as shown. Incubation medium was MS medium (*None*), 200 mM sucrose (*Suc*), 200 mM glucose (*Glc*), 200 mM mannitol (*Man*). *Panel D*, accumulation of starch in detached leaves. Starch was stained with iodine by the method of Johnson and Ryan (1990).

DISCUSSION

This is the first report of the complete purification and characterization of D-enzyme from a plant. The enzyme catalyzes glucan transfer from one 1,4-α-D-glucan molecule to another, or to glucose. Maltotriose and larger molecules, including amylose and amylopectin, can serve as donors (Fig. 2, *A* and *B*). Glucose and maltooligosaccharides can serve as acceptors (Fig. 2, *A* and *B*). To understand fully the function of D-enzyme, it may be important to know whether starch molecules are effective acceptors for glucan transfer. We observed that relatively high molecular weight molecules are produced by the action of D-enzyme on maltopentaose (Fig. 2*A*), showing that acceptors can be large oligosaccharides. When the products of this reaction were separated by gel filtration (results not shown) molecules equivalent in size to short chain amylose were observed. Furthermore, when the products of action of D-enzyme on short chain amylose were also analyzed by gel filtration, evidence for the production of larger molecules was obtained (results not shown). These techniques are not sufficiently sensitive to resolve changes in chain length in high molecular weight starch molecules, so the effectiveness of amylose and amylopectin as acceptors remains to be determined. Similarly, we do not know how effective starch molecules are as donors for glucan transfer to other starch molecules.

With the available information, it is possible to propose at least three different functions for D-enzyme. First, it may act during starch breakdown to convert small oligosaccharides into larger molecules upon which starch phosphorylase can act (Lee and Whelan, 1971). Second, it may change the structure of starch molecules and grain architecture by modifying chain lengths. Third, it may generate from starch and

glucose, oligosaccharides which can serve either as primers for new starch synthesis, or as substrates for starch phosphorylase or amylases. The apparent plastidic location of D-enzyme and its pattern of synthesis in potato tubers and leaves are consistent with any of these possible functions (see below), so final elucidation of the function of this enzyme may await the genetic manipulation analysis which is now made possible by molecular cloning of the cDNA.

The native enzyme from potato tubers consists of a single polypeptide of M_r 59,500. This enzyme is apparently made as a higher molecular weight precursor of 65,000. This is entirely consistent with the observation that the enzyme is found in extracts of chloroplasts from *Pisum sativum* (Kakefuda *et al.*, 1986), spinach (Okita *et al.*, 1979; Stitt, 1984), and *Arabidopsis* leaves (Lin *et al.*, 1988). In the last case D-enzyme was found exclusively in the chloroplast fraction. We propose therefore that the amino-terminal amino acid pre-piece functions as a transit peptide for plastid targeting, although a chloroplast or amyloplast location for potato D-enzyme has not yet been reported. The putative transit peptide has many features in common with known transit peptides, including a high proportion of hydroxyl and basic amino acids, few acidic amino acids, and similarity to a consensus motif at the processing site (Gavel and von Heijne, 1990). In the potato tuber the protein is presumably targeted to the amyloplast. We do not know if a single D-enzyme is synthesized throughout the potato plant or if there are isoforms which might be specific to particular organs. However, it is well established that transit peptides can function interchangeably between chloroplasts and amyloplasts (de Boer *et al.*, 1988; Klösgen *et al.*, 1989; Klösgen and Weil, 1991) so a single D-enzyme is possible. Preliminary analysis of four tuber cDNA clones found no evidence for sequence heterogeneity.

Expression of D-enzyme gene is observed in all organs examined, as would be expected of a gene controlling starch metabolism. Expression is regulated during potato tuber development and in leaves in response to light and sucrose. Surprisingly, D-enzyme mRNA accumulates under circumstances when starch biosynthesis is most active (in developing tubers and in illuminated or sucrose-treated leaves) but declines in amount under conditions when starch is broken down (cold-stored tubers, sprouting tubers, and dark-incubated leaves). These observations appear to contradict the widely held view that this enzyme is involved in starch breakdown and may suggest a function in starch synthesis. However, the gene encoding potato L-type starch phosphorylase shows a similar pattern of expression in developing tubers (Mori *et al.*, 1991). It is possible therefore that starch breakdown is controlled mainly through post-transcriptional processes, such as enzyme synthesis, activation, or substrate availability, although we were unable to identify molecules which could regulate D-enzyme activity. It will be necessary in the future to investigate carefully the changes in amount and specific activity of these enzymes during starch metabolism. The possible involvement of hydrolytic enzymes in potato starch breakdown also requires careful examination (Krugar, 1990). In this context it would also be of value to examine D-enzyme synthesis and activity in a tissue such as cereal endosperm, in which amylases are thought to be the major route of starch breakdown.

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Potato D-enzyme Catalyzes the Cyclization of Amylose to Produce Cycloamylose, a Novel Cyclic Glucan*

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Takeshi Takaha‡§¶, Michiyo Yanase§, Hiroki Takata§, Shigetaka Okada§, and Steven M. Smith‡

From the ‡Institute of Cell and Molecular Biology, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, United Kingdom and the §Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Utajima, Nishiyodogawa-ku, Osaka 555, Japan

Potato D-enzyme was purified from recombinant *Escherichia coli*, and its action on synthetic amylose (average M_r of 320,000) was analyzed. D-enzyme treatment resulted in a decrease in the ability of the amylose to form a blue complex with iodine. Analysis of the products indicated that the enzyme catalyzes an intramolecular transglycosylation reaction on amylose to produce cyclic α-1,4-glucan (cycloamylose). Confirmation of the cyclic structure was achieved by demonstrating the absence of reducing and nonreducing ends, resistance to hydrolysis by glucoamylase (an exoamylase), and by "time of flight" mass spectrometry. The degree of polymerization of cycloamylose products was determined by time of flight mass spectrometry analysis and by high-performance anion-exchange chromatography following partial acid hydrolysis of purified cycloamylose molecules and was found to range from 17 to several hundred. The yield of cycloamylose increased with time and reached >95%. D-enzyme did not act upon purified cycloamylose, but if glucose was added as an acceptor molecule, smaller cyclic and linear molecules were produced. The mechanism of the cyclization reaction, the possible role of the enzyme in starch metabolism, and the potential applications for cycloamylose are discussed.

D-enzyme (disproportionating enzyme or 4-α-glucanotransferase, EC 2.4.1.25) was first found in potato tubers by Peat *et al.* (1), but has since been found in many plant tissues (2). The enzyme is known to catalyze glucan transfer from one α-1,4-glucan molecule to another or to glucose. Malto-oligosaccharides have been shown to be effective donors and malto-oligosaccharides and glucose to serve as acceptors *in vitro*. A maltosyl group has been recognized as the major transferred unit from the donor molecule (3, 4). It has therefore been proposed that the enzyme could be involved in starch breakdown to produce malto-oligosaccharides upon which starch phosphorylase can act (2, 5). Both enzymes are found in plastids (6), and a similar function has been proposed for *Escherichia coli* where D-enzyme (amylomaltase) and maltodextrin phosphorylase are encoded by genes of the *malA* operon and are involved in exogenous malto-oligosaccharide utilization (7). However, in plants, there is a further possibility that high molecular weight starch might be the real substrate of the enzyme. In this case, we could consider the possibility that D-enzyme may change starch molecule structure and grain architecture by transferring malto-oligosyl units from one chain to another (8). It is known that the enzyme can use soluble starch (4) or amylopectin (2) as the donor molecule, but it is not known if the enzyme can use high molecular weight starch as an acceptor molecule. Thus, further investigations of the action of the enzyme on high molecular weight starch are important in order to obtain a better understanding of the role of D-enzyme in starch metabolism.

We reported the purification of D-enzyme from potato tuber extracts and the isolation of a cDNA clone from potato tuber RNA (8). Northern blot analysis showed that D-enzyme mRNA is present in leaves, stems, roots, and stolons, but is most abundant in developing and mature tubers (8). D-enzyme mRNA accumulates under circumstances in which starch biosynthesis is most active, similar to that of enzymes of starch synthesis, including ADP-glucose pyrophosphorylase (9), granule-bound starch synthase (10), and starch branching enzyme (11), which may suggest a role for D-enzyme in starch synthesis (8). Here, we report that D-enzyme does use high molecular weight amylose as the donor or acceptor molecule and catalyzes a novel cyclization reaction.

EXPERIMENTAL PROCEDURES

Materials—Synthetic amylose (average molecular masses of 5, 10, 30, 70, 110, 320, and 1000 kDa) was obtained from Nakano Vinegar Co., Ltd. (Aichi, Japan). Isoamylase and pullulanase were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Bacterial saccharifying α-amylase was obtained from Nagase Biochemicals (Kyoto, Japan). Glucoamylase from *Rizopus* sp. was purchased from Toyobo Co., Ltd. (Osaka, Japan).

Preparation of Recombinant Potato D-enzyme from E. coli—*E. coli* cells carrying the plasmid pKK233-DPE (8) were cultured in LB medium at 37 °C until late log phase, and then the inducer isopropyl-1-thio-β-D-galactopyranoside (1 mM) was added and incubated for 6 h at 15 °C. Cells were harvested by centrifugation and disrupted by sonication in 20 mM Tris-Cl (pH 7.5) containing 5 mM 2-mercaptoethanol (buffer A) at 4 °C. This crude extract was centrifuged, and the supernatant was filtered through a 0.45-μm membrane and then loaded onto a Q-Sepharose fast flow column (16 × 100 mm; Pharmacia Biotech Inc.) and washed with 150 mM NaCl in buffer A. D-enzyme was eluted with buffer A containing 450 mM NaCl and dialyzed against buffer A. Concentrated Tris buffer, ammonium sulfate, and 2-mercaptoethanol were added to the dialysate to give final concentrations of 50 mM Tris-Cl (pH 7.5), 500 mM ammonium sulfate, and 5 mM 2-mercaptoethanol. The solution was loaded onto a phenyl-TOYOPEARL 650M column (10 × 100 mm; TOSOH, Tokyo, Japan) and eluted with a linear gradient of 500 to 0 mM ammonium sulfate in buffer A. Active fractions were pooled and dialyzed against buffer A. The dialysate was loaded onto a Resource Q column (6 ml; Pharmacia Biotech Inc.). The enzyme was eluted with a linear gradient of 150–400 mM NaCl in buffer A. Active fractions were concentrated with an Amicon Centricon 30 and dialyzed against 20 mM sodium citrate buffer (pH 7.0).

Assay of D-enzyme—D-enzyme activity was assayed in a 100-μl reaction mixture containing 100 mM Tris-Cl (pH 7.0), 1% (w/v) maltotriose, and enzyme. The reaction mixture was incubated at 37 °C for 10

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¶ To whom correspondence should be addressed: Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Utajima, Nishiyodogawa-ku, Osaka 555, Japan. Tel.: 81-6-477-8425; Fax: 81-6-477-6850; E-mail: glicobio@tau.bekkoame.or.jp.

min, and the reaction was terminated by immersing the reaction tubes in boiling water for 3 min. Released glucose was measured by the glucose oxidase method (12). One unit of activity is defined as the amount of enzyme that produces 1 μmol of glucose/min under these assay conditions.

Preparation of Amylose Solution—Synthetic amylose (20 mg) was dissolved in 2 ml of 1 N NaOH solution and then neutralized by adding 4 ml of distilled water, 2 ml of 1 M sodium citrate buffer, and 2 ml of 1 N HCl. The solution was used immediately after neutralization.

Absorption Spectrum of Glucan-Iodine Complex—One-hundred μl of 0.1% (w/v) glucan solution was mixed with 2 ml of iodine reagent, and the absorption spectrum was monitored using a Shimazu UV-240 spectrophotometer. Iodine reagent was made daily from 0.5 ml of iodine stock solution (0.26 g of I_2 and 2.6 g of KI in 10 ml of water) mixed with 0.5 ml of 1 N HCl and diluted to 130 ml with distilled water.

Gel Filtration Chromatography—Gel filtration chromatography was carried out using a Superose 6 prep grade column (10 × 300 mm; Pharmacia Biotech Inc.) plus a Superdex 30 column (10 × 300 mm; Pharmacia Biotech Inc.) with a flow rate of 1 ml/min. The eluent used was 150 mM sodium acetate. The molecular mass of the glucan was estimated using a standard curve produced with synthetic amylose with an average molecular mass of 5, 10, 30, 70, 320, or 1000 kDa as a standard.

Determination of Yield of Glucoamylase-resistant Molecules—The amount of glucoamylase-resistant molecules in the presence of linear glucan was determined as follows. The glucan (100 μg) was incubated either with glucoamylase (10 units) only or with glucoamylase (10 units) and α-amylase (10 units) in 100 mM acetate buffer (pH 5.5) for 1 h at 40 °C. After terminating the reaction by boiling for 5 min, released glucose was measured by the glucose oxidase method (12). The amount of glucoamylase-resistant molecules was calculated by subtracting the amount of glucose released by glucoamylase and α-amylase from that released by glucoamylase only.

High-performance Anion-exchange Chromatography (HPAEC)[1]—HPAEC was carried out with a Dionex DX-300 system with a pulsed amperometric detector (Model PAD-II, Dionex Corp., Sunnyvale, CA). For analytical purposes, a Carbopac PA-100 column (4 × 250 mm) was used. A sample (25–250 μl) containing 25–100 μg of glucan was injected and eluted with a gradient of sodium acetate (0–2 min, 50 mM; 2–37 min, increasing from 50 to 350 mM with installed gradient program 3; 37–45 min, increasing from 350 to 850 mM with installed gradient program 7; and 45–47 min, 850 mM) in 150 mM NaOH with a flow rate of 1 ml/min.

Quantitation of Reducing and Nonreducing Ends of Glucan—Reducing and nonreducing ends were quantitated using a modified Park-Johnson method (see Ref. 13) and the rapid Smith degradation method (see Ref. 14), respectively.

Preparation of Glucoamylase-resistant Molecules and Their Size Fractionation by Gel Filtration Chromatography—A reaction mixture (10 ml) containing 20 mg of synthetic amylose AS-320 (Nakano Vinegar Co., Ltd.), 50 mM sodium citrate buffer (pH 7.0), 100 mM NaCl, and 68 units of purified D-enzyme from recombinant *E. coli* was incubated at 30 °C for 18 h and then boiled for 5 min to terminate the reaction. The denaturated enzyme was removed by centrifugation, and the supernatant was incubated with 100 units of glucoamylase for 4 h at 40 °C. The resultant glucoamylase-resistant glucan was precipitated with 10 volumes of ethanol and lyophilized. The pellet of glucoamylase-resistant glucan was dissolved in 250 μl of distilled water and then size-fractionated by gel filtration chromatography using a Superdex 30 column (10 × 300 mm) with a flow rate of 0.5 ml/min.

Purification of Cyclic α-1,4-Glucan with Single DP by HPAEC and Its Partial Acid Hydrolysis—The size-fractionated glucoamylase-resistant molecule (1 mg) was injected onto a Carbopac PA-1 column (9 × 250 mm) and eluted with a gradient of sodium acetate (0–2 min, 230 mM; 2–37 min, increasing from 230 to 280 mM with installed gradient program 6; 37–45 min, 280 mM; and 45–47 min, 850 mM) in 150 mM NaOH with a flow rate of 5 ml/min. The eluate from the column was divided for pulsed amperometric detection and for sample preparation. The eluate for each peak was collected, neutralized with 1 N HCl, precipitated with 10 volumes of ethanol, and lyophilized. Purified glucan (~50 μg) for each peak was resuspended in 20 μl of distilled water. A 10-μl aliquot of the glucan solution was mixed with 10 μl of 0.2 N HCl and incubated for 15 min at 100 °C. Acid hydrolysis was terminated by adding 240 μl of ice-cold 150 mM NaOH and was immediately analyzed by HPAEC with a Carbopac PA-100 column.

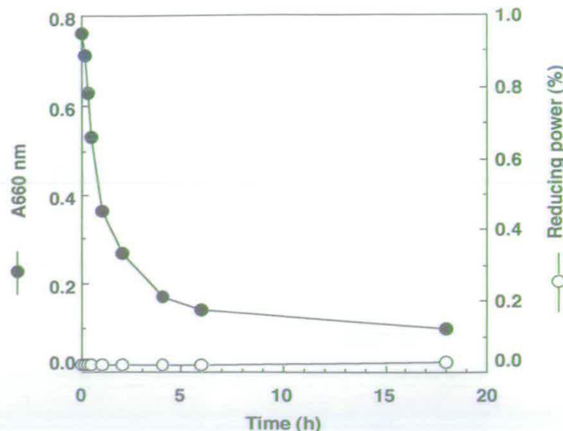

FIG. 1. **Effect of D-enzyme on formation of amylose-iodine complex.** A reaction mixture (20 ml) containing 40 mg of synthetic amylose AS-320, 50 mM sodium citrate buffer (pH 7.0), 100 mM NaCl, and 136 units of purified D-enzyme from recombinant *E. coli* was incubated at 30 °C. At the indicated time points, 1 ml of the reaction mixture was removed, boiled for 5 min to stop the reaction, and then centrifuged. Fifty μl of the supernatant was mixed with 50 μl of distilled water and 2 ml of iodine reagent to measure the absorbance at 660 nm, and 200 μl was used for reducing power measurement by a modified Park-Johnson method (see Ref. 13). Reducing power when all the amylose was broken down to glucose was defined as 100%.

"Time of Flight" Mass Spectrometry (TOF-MS)—The molecular mass of the glucan mixture was analyzed by a Kompact Maldi I TOF-MS system (Shimazu Co., Kyoto, Japan).

RESULTS

Action of D-enzyme on Synthetic Amylose—Synthetic amylose was chosen to investigate the action of D-enzyme on high molecular weight starch since it is a linear α-1,4-glucan free from α-1,6-branches or other modifications. Purified potato D-enzyme from recombinant *E. coli* was incubated with synthetic amylose AS-320 (average M_r of 320,000). During the reaction, the ability of amylose to form a blue complex with iodine (the blue value) decreased, and a brown product resulted instead. To understand how this decrease in the blue value was caused by D-enzyme, samples were taken at time points during the reaction, and the blue value (A_{660}) and reducing power were measured. As shown in Fig. 1, A_{660} decreased rapidly during the initial few hours, but more slowly subsequently. If this decrease in the blue value was caused by the hydrolysis of amylose, an increase in reducing power should be expected. However, no increase in reducing power was detected. These results indicate that the decrease in the blue value was not caused by hydrolytic activity on the amylose molecule. It is well known that a similar change in the absorption spectrum of amylose-iodine complexes can be produced by starch branching enzyme introducing branches into the amylose molecule. We measured the number of α-1,6-linkages during the incubation period by quantitating reducing sugars after isoamylase treatment (13), but none were detected.

To determine how the decrease in the blue value is caused by D-enzyme, possible structural changes in the amylose molecule were analyzed by gel filtration chromatography. As shown in Fig. 2*A*, the narrow size distribution of amylose AS-320 was changed to a broad distribution during the initial few minutes, but subsequently, the products showed a narrow size distribution in the low molecular weight region (average M_r of 15,000). Similar results were obtained using other high molecular weight amyloses (AS-1000 and AS-110) as substrates, in each case yielding products with a M_r of 15,000 (data not shown). Since no increase in reducing power was detected, the most

[1] The abbreviations used are: HPAEC, high-performance anion-exchange chromatography; DP, degree of polymerization; TOF-MS, time of flight mass spectrometry.

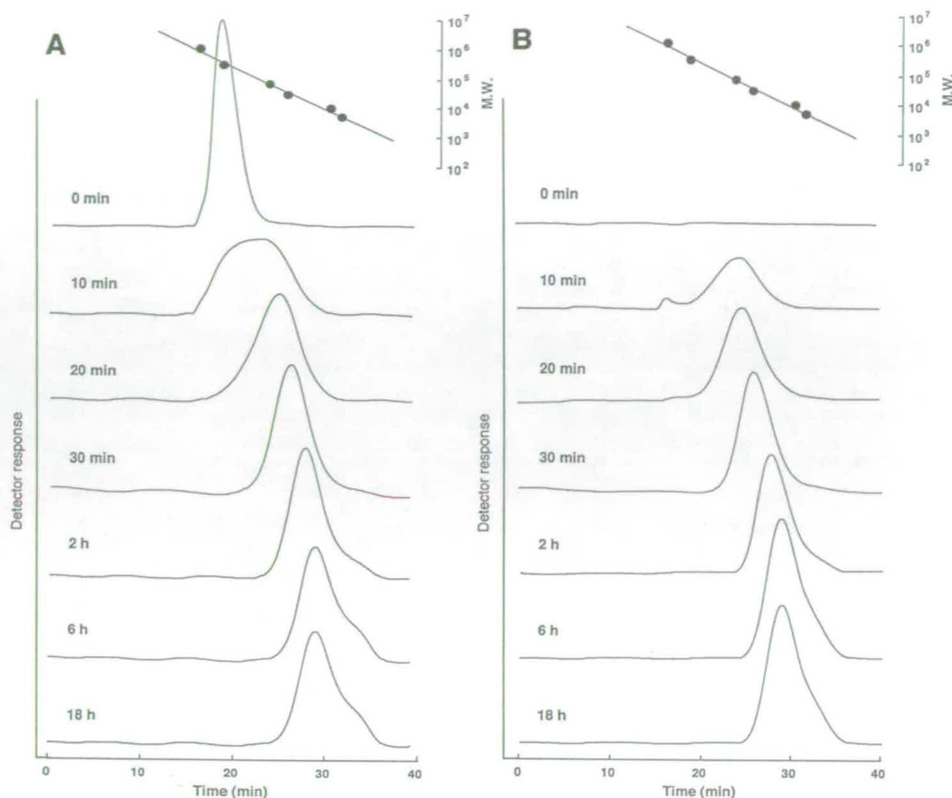

FIG. 2. **Gel filtration chromatography of products of D-enzyme action on amylose AS-320.** Two-hundred fifty μl of the supernatant derived from the experiment shown in Fig. 1 was analyzed before (*A*) and after (*B*) glucoamylase treatment by gel filtration chromatography using a Superose 6 prep grade column plus a Superdex 30 column with a flow rate of 1.0 ml/min. The standard curve for the molecular mass was produced using synthetic amylose with an average molecular mass of 5, 10, 30, 70, 320, or 1000 kDa as a standard.

likely explanation for this observation is that D-enzyme catalyzes an intramolecular transglycosylation reaction (cyclization reaction) to produce cyclic α-1,4-glucans.

The possibility that D-enzyme could have catalyzed the formation of cyclic α-1,4-glucans was examined by glucoamylase treatment. Glucoamylase is an exo-type amylase and hydrolyzes both α-1,4- and α-1,6-linkages in starch to produce glucose from the nonreducing end of the substrate. Thus, linear or branched glucans are completely broken down to glucose by glucoamylase. However, glucan with cyclic structure should be resistant to glucoamylase. The samples shown in Fig. 2*A* were treated with glucoamylase, and the resultant glucoamylase-resistant glucan was precipitated with ethanol and then analyzed with the same gel filtration column (Fig. 2*B*). The peak of intact amylose AS-320 (0 min) was completely hydrolyzed by glucoamylase, but molecules produced by the action of D-enzyme were resistant to glucoamylase. The amount of glucoamylase-resistant molecules and their average molecular weights are shown in Fig. 3. The yield of glucoamylase-resistant molecules increased to its maximum level (>95%) within 30 min and then remained constant. The average molecular weight of glucoamylase-resistant molecules estimated by gel filtration was initially ~70,000, but decreased with time to ~15,000. The presence of these glucoamylase-resistant molecules strongly suggested that D-enzyme had catalyzed the cyclization of amylose AS-320 and produced cyclic α-1,4-glucans.

Analysis of Structure of Glucoamylase-resistant Molecules—Further structural analysis of the final products of D-enzyme action on amylose AS-320 was carried out by HPAEC. The elution pattern of the products after 18 h of D-enzyme treatment is shown in Fig. 4*A*, and α-1,4-glucan standards are shown in Fig. 4*D*. The minor peaks that eluted between 3 and 17 min had the same retention times as linear α-1,4-glucans with a DP of between 1 and 14, but the major peaks that eluted after 17 min had different retention times than α-1,4-glucans. The nature of the products was first analyzed by treatment with glucoamylase. The minor products of D-enzyme reaction

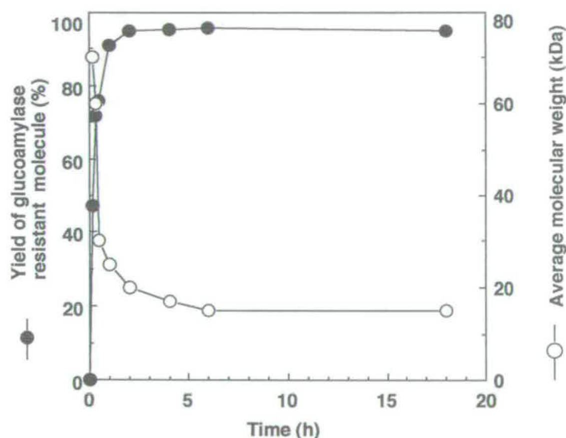

FIG. 3. **Yield and average molecular weight of glucoamylase-resistant molecules produced by D-enzyme.** The yield of glucoamylase-resistant molecules was measured as described under "Experimental Procedures." The average molecular weight of glucoamylase-resistant molecules was estimated by gel filtration chromatography using synthetic amylose with different average molecular masses as a standard (see Fig. 2*B*).

that eluted between 3 and 17 min were completely hydrolyzed to glucose by glucoamylase (Fig. 4*B*). However, the major products with retention times >17 min were resistant to glucoamylase. It should also be noted that no peaks corresponding to cyclodextrins (15) were detected in the glucoamylase-resistant molecules (Fig. 4*B*). The products were next analyzed by treatment with α-amylase, an endoamylase that hydrolyzes α-1,4-glucans to produce only glucose and maltose. The products of D-enzyme action were completely hydrolyzed to glucose and maltose (Fig. 4*C*), indicating that such products are all α-1,4-glucans without any modification or branching.

The glucoamylase-resistant products were next purified from glucose and then size-fractionated by gel filtration chromatography. The putative cyclic α-1,4-glucans in each fraction were

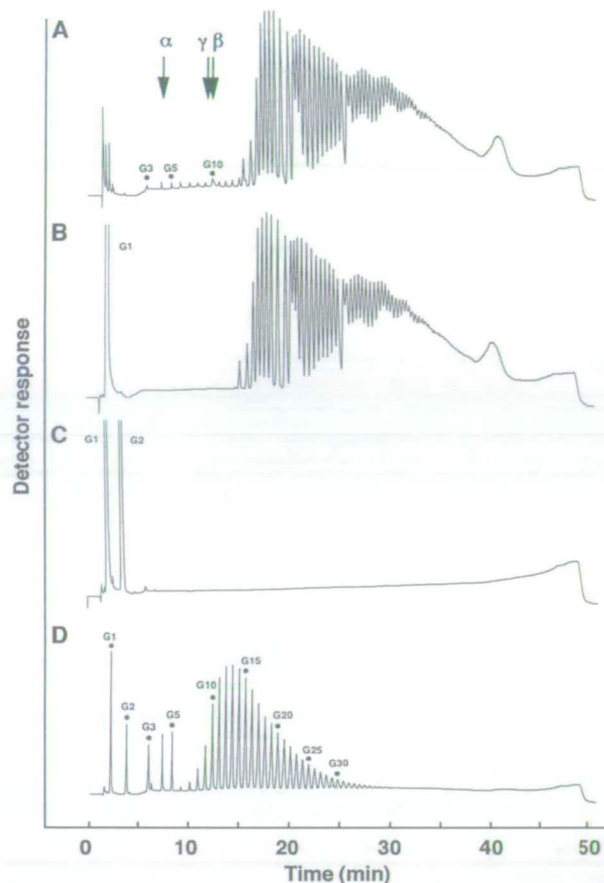

FIG. 4. **HPAEC analysis of products of D-enzyme action on amylose AS-320.** The products of D-enzyme treatment (18 h) of amylose AS-320 were analyzed by HPAEC using the conditions described under "Experimental Procedures." Fifty μg of glucan before any treatment (*A*), after glucoamylase treatment (*B*), and after α-amylase treatment (*C*) was analyzed. Short-chain amylose was mixed with glucose and malto-oligosaccharides and used as an α-1,4-glucan marker (*D*). Positions where α-, β-, and γ-cyclodextrins were eluted are indicated by *arrows*. Numbers above and beside peaks (*G1*, *G5*, etc.) indicate the DPs of the products. The amylase treatments were carried out at pH 5.5 and 40 °C. The amounts of glucoamylase and α-amylase used were 50 and 10 units/mg of glucan, respectively.

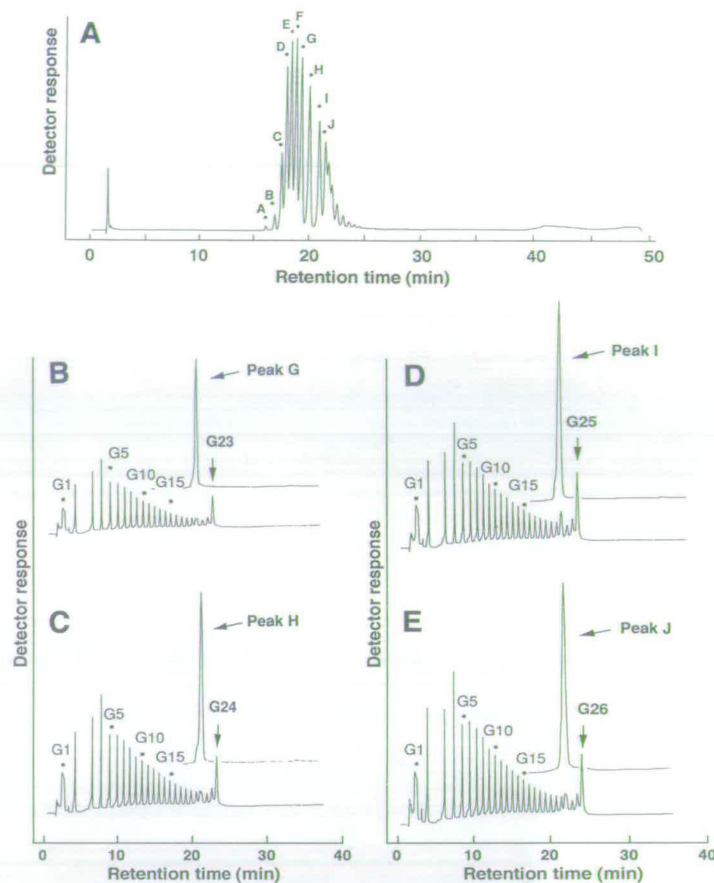

FIG. 5. **Partial acid hydrolysis of glucoamylase-resistant molecules.** Glucoamylase-resistant molecules were size-fractionated by gel filtration chromatography as described under "Experimental Procedures," and the fractions containing the smallest glucans were analyzed by HPAEC (*A*). Glucoamylase-resistant peaks G–J were purified by HPAEC and partially hydrolyzed as described under "Experimental Procedures." The elution profiles of each purified peak before (*upper trace*) and after (*lower trace*) partial acid hydrolysis are shown (*B–E*). Numbers above peaks (*G1*, *G5*, etc.) indicate the DPs of the products, with the largest product indicated by an *arrow*.

precipitated with ethanol and then lyophilized. Quantitation of reducing and nonreducing ends was carried out, but none were detected (data not shown), consistent with the proposed cyclic structure. Further evidence for the cyclic nature of the products of D-enzyme action was obtained by partial acid hydrolysis. The gel filtration fractions that contained the lower molecular weight glucoamylase-resistant glucans were next separated by HPAEC (Fig. 5*A*). Peaks G–J were purified by further HPAEC and then partially hydrolyzed with 0.1 N HCl. Products from peak G hydrolysis eluted as linear molecules with a DP of 1–23 (Fig. 5*B*). These products were hydrolyzed to glucose by glucoamylase (data not shown). The largest product of partial acid hydrolysis, with a DP of 23 (G23), is assumed to have resulted from hydrolysis of one glucosidic linkage of the peak G molecule. Similar results were obtained for all other peaks, except that the DP of the largest linear product was 1 unit larger for each successive peak (Fig. 5, *C–E*). The retention time of each peak was less than that of the largest linear product.

Final confirmation of the cyclic nature of the glucoamylase-resistant products of D-enzyme action and determination of their molecular masses were obtained by TOF-MS, which can determine the molecular mass not only of single molecules, but also of several molecules in a mixture. A glucan with a DP of n in any non-cyclic structure should have a molecular mass of $162n + 18$, whereas a glucan with a DP of n in a cyclic structure should have a mass of $162n$. The fraction containing the smallest glucoamylase-resistant molecules (Fig. 5*A*) was subjected to TOF-MS (Fig. 6). Several peaks were obtained in the mass spectrometry spectrum, and the molecular masses agreed with the theoretical values for cyclic glucans with DPs of 17 and greater, but not with those for linear glucans. From this and previous results, we conclude that the smallest glucoamylase-resistant molecule (peak A) is cyclic α-1,4-glucan with a DP of 17 (cG17) and that the consecutive peaks (B, C, D, etc.) are cyclic glucans with DPs of 1 unit longer for each successive peak (cG18, cG19, cG20, etc.). Therefore, our results show that D-enzyme catalyzes an intramolecular transglycosylation reaction on amylose to produce cyclic α-1,4-glucans (cycloamylose) with DPs ranging from a minimum of 17 (peak A) to a few hundred (Fig. 3).

Action of D-enzyme on Cyclic α-1,4-Glucan—Figs. 2*B* and 3 indicated that high molecular weight cyclic α-1,4-glucans initially produced by D-enzyme were converted into lower molecular weight cyclic α-1,4-glucans by prolonged D-enzyme action. The fact that reducing power was not increased during the reaction (Fig. 1) suggested that this conversion did not involve hydrolysis, but was brought about by the transglycosidic action of D-enzyme, in which case an acceptor molecule should be required for this conversion. To test this hypothesis, high mo-

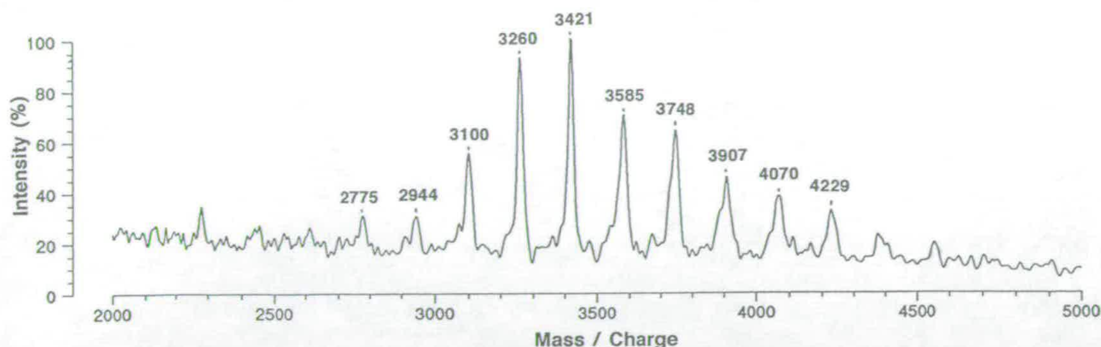

FIG. 6. **TOF-MS analysis of glucoamylase-resistant molecules.** Size-fractionated glucoamylase-resistant molecules containing the smallest glucans (shown in Fig. 5*A*) were subjected to TOF-MS analysis. Numbers above each peak indicate the molecular mass of the glucan + 23 (sodium ion).

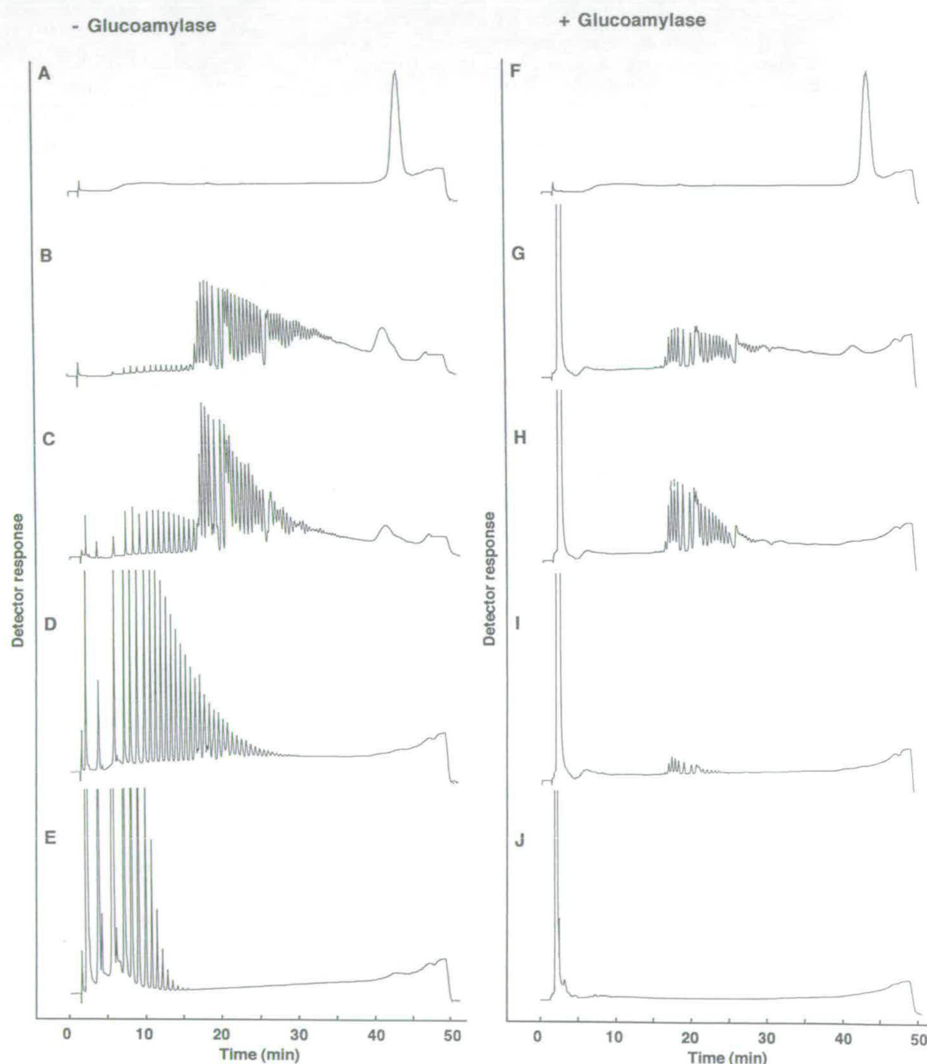

FIG. 7. **Analysis of action of D-enzyme on cyclic α-1,4-glucan in presence and absence of acceptor molecule.** Size-fractionated cyclic α-1,4-glucan (1 mg) with an average molecular mass of 30 kDa was treated with D-enzyme (1 unit) in the absence of glucose (*A* and *F*) or in the presence of glucose at a weight ratio (glucose/cyclic glucan) of 0.0005 (*B* and *G*), 0.005 (*C* and *H*), 0.05 (*D* and *I*), or 0.5 (*E* and *J*). This corresponds to glucose concentrations of 0.1–110 mM. The reaction mixtures were incubated at 30 °C for 18 h and then boiled to terminate the reaction. The products (100 μg) were analyzed by HPAEC before (*A–E*) and after (*F–J*) glucoamylase treatment.

lecular weight cyclic α-1,4-glucan (average M_r of 30,000) prepared by gel filtration was incubated with D-enzyme in the presence or absence of glucose as an acceptor molecule. No lower molecular weight products were produced in the absence of glucose (Fig. 7*A*), but lower molecular weight products were produced in the presence of glucose (Fig. 7, *B–E*). As the proportion of glucose was increased, the amount of lower molecular weight molecules produced also increased (Fig. 7, *B–E*). The presence of lower molecular weight cyclic α-1,4-glucan was confirmed by analyzing the glucoamylase-resistant molecules in each reaction (Fig. 7, *F–J*). As the proportion of glucose was increased, the amount of cyclic α-1,4-glucan decreased (Fig. 7, *G–J*). This result is dependent on the ratio of glucose to amylose, not on concentrations. These results are consistent with a transglycosidic mode of conversion of high molecular weight cyclic α-1,4-glucans into lower molecular weight cyclic α-1,4-glucans.

DISCUSSION

D-enzyme has previously been shown to catalyze a transglycosylation reaction on malto-oligosaccharides, and the action of this enzyme on small oligosaccharides has been extensively

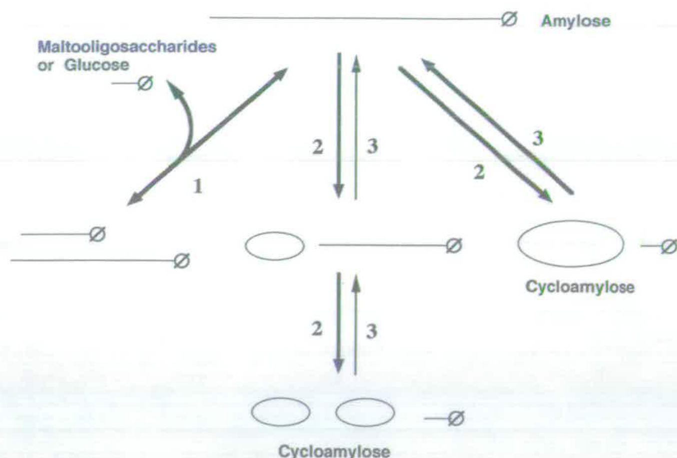

FIG. 8. **Diagrammatic representations of action of D-enzyme on amylose and cycloamylose.** *Lines* and *circles* indicate α-1,4-glucan chains, where the relative length represents their relative DP. Ø, glucosyl residue at reducing end. *Reaction 1* is the disproportionation reaction (intermolecular transglycosylation). *Reaction 2* is the cyclization reaction (intramolecular transglycosylation). *Reaction 3* is the transglycosidic linearization reaction (intermolecular transglycosylation).

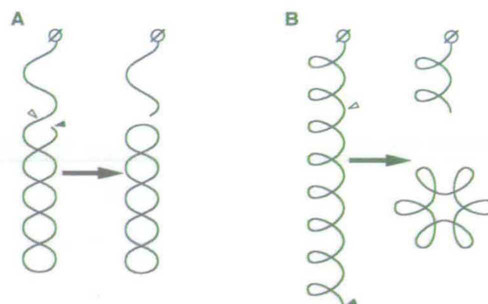

FIG. 9. **Diagrammatic representations of cyclization of amylose in antiparallel double (*A*) and single (*B*) helix configurations.** Ø, glucosyl residue at reducing end. *Open* and *closed arrowheads* indicate the sites attacked by D-enzyme (donor site) and those to which the α-1,4-glucan chain will be transferred (acceptor site), respectively.

analyzed (3, 4). However, little is known about the action of this enzyme on amylose or amylopectin, although it has been demonstrated that malto-oligosaccharide units (a maltose unit is preferred) can be transferred from starch to glucose (2, 4). The present results clearly demonstrate novel activities of D-enzyme. First, it can catalyze an intramolecular transglycosylation reaction on high molecular weight amylose to produce cyclic α-1,4-glucans (cycloamylose) with a DP ranging from 17 to several hundred. Second, amylose can serve as donor and acceptor. Third, very long α-1,4-glucan units can be transferred by the enzyme. Fourth, D-enzyme catalyzes the transglycosidic linearization of cycloamylose when an acceptor is present. These activities of D-enzyme are explained in Fig. 8. The reaction starts with D-enzyme attacking an α-1,4-linkage. The enzyme then transfers the newly formed reducing end of the substrate either to the nonreducing end of a separate linear acceptor molecule or glucose (the intermolecular transglycosylation or disproportionation reaction) or to its own nonreducing end (the intramolecular transglycosylation or cyclization reaction). The reversibility of these reactions allows high molecular weight cyclic molecules to be linearized again by transglycosylation and lower molecular weight cyclic molecules to be produced subsequently (Fig. 8). Apparently, the equilibrium tends toward the formation of cycloamylose with a M_r of ~15,000 (DP of 90), as shown in Fig. 3.

Cyclodextrin glucanotransferase also catalyzes cyclization and disproportionation reactions on α-1,4-glucans (15). The transglycosidic linearization of cyclodextrin in the presence of a suitable acceptor has also been demonstrated (the "coupling reaction") (15). In all these respects, D-enzyme and cyclodextrin glucanotransferase seem to catalyze the same reaction, but the major difference is the DP of the cyclic α-1,4-glucans produced. Cyclodextrin glucanotransferase produces cyclodextrins with DPs of 6, 7, or 8. Larger cyclodextrins with DPs of 9–13 have been reported, but only in trace amounts (16). D-enzyme produces cycloamylose with DPs ranging from 17 to several hundred. This observation suggests that there may be fundamental differences between cyclodextrins and cycloamylose and the enzymes that act upon them.

Cyclic α-1,4-glucans can potentially adopt antiparallel double helix, single helix, or nonhelical conformations. Cyclodextrins are known to adopt a nonhelical structure (17). Energy calculations of cyclic α-1,4-glucans in the nonhelical conformation indicated that cyclodextrin with a DP of 6 has the minimum energy and that as cyclodextrin size increases, so does the energy (18). The observation that cyclodextrins with DPs of 6 and 7 are the major products of cyclodextrin glucanotransferase activity agrees well with such calculations (19). It has been reported that linear amylose can occur in a double-stranded or single-stranded helix conformation (20). Fig. 9 shows that either conformation in linear amylose could potentially allow cyclization to form cycloamylose in either configuration. The Monte Carlo simulation suggested that the antiparallel double helix structure is the most likely conformation of amylose in solution (21), and crystallographic analysis has shown that linear amylose with a DP of 6 adopts a left-handed antiparallel double helix conformation (22, 23). It can be seen how such a conformation would readily allow cyclization by D-enzyme since donor and acceptor sites of amylose are juxtaposed (Fig. 9*A*) and so may be readily accommodated by the active site of the enzyme. Energy minimization calculations suggest that cycloamylose in the antiparallel double helix conformation is more stable than in the single helix configuration.[2] We therefore favor the view that cycloamylose adopts the antiparallel double helix structure, but confirmation awaits structural analysis.

The function of D-enzyme in plants remains unknown. Preliminary results[3] showed that D-enzyme can also catalyze intramolecular transglycosylation reactions on amylopectin *in vitro*. A role for D-enzyme in starch breakdown can be considered in which linear or cyclic glucans are produced as substrates for hydrolytic or phosphorolytic enzymes. We have not yet been able to detect cycloamylose *in vivo*, which could be explained if it has a short half-life. However, cycloamylose may not be produced *in vivo* if the ratio of acceptor (*e.g.* glucose) to amylose is high (Fig. 7), but we have no information on such a ratio *in vivo*. Alternatively, D-enzyme could modify starch structure through its glucanotransferase activity. The function may be revealed when mutants lacking D-enzyme can be obtained.

Due to the high efficiency of cyclization of amylose by D-enzyme *in vitro* and the production of recombinant D-enzyme in *E. coli*, the large-scale production of cycloamylose is feasible. Preliminary experiments have shown that cycloamylose has several interesting properties. It is nonreducing, is highly soluble in cold water, and can form inclusion complexes with several inorganic and organic compounds (data not shown). Cycloamylose may have different dimensions and tertiary structure than cyclodextrins, so that different specificities for

[2] J. Shimada, personal communication.

[3] T. Takaha, M. Yanase, H. Takata, S. Okada, and S. M. Smith, unpublished data.

guest molecules can be anticipated. Therefore, there is great potential for the exploitation of cycloamylose in chemical, pharmaceutical, and food industries to safely achieve the solubilization, increased stability, sequestration, or altered reactivity of molecules with which it can form inclusion complexes.

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