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Isoamylase and its Industrial Significance in the Production of Sugars from Starch

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Introduction

Debranching enzymes are important in the basic and applied science of starch. The usual composition of starch is about 80% amylopectin and 20% amylose. Amylopectin is partially hydrolysed by α-amylase because its branch points with (1,6-α)-glucosidic linkages are resistant to attack by the usual α-amylase; only α-amylase (EC 3.2.1.1) from *Thermoactinomyces vulgaris* has been shown to hydrolyse both (1,6-α)-glucosidic linkages and (1,4-α)-glucosidic linkages (Sakano *et al.*, 1982). Glucoamylase (exo-1,4-α-D-glucosidase, EC 3.2.1.3), which is capable of producing glucose from starch, can hydrolyse (1,6-α)-glucosidic linkages in amylopectin, although slowly. β-Amylase (EC 3.2.1.2) forms maltose from the non-reducing point of the chain in amylopectin but the hydrolysis stops near the branching points of the chain, leaving β-amylase limit dextrin (β-limit dextrin). Thus, the ability to produce maltose or glucose from starch can be improved by using a debranching enzyme.

Debranching enzymes are classified as direct or indirect (Lee and Whelan, 1971). The former, which can attack amylopectin and glycogen directly, are principally divided into isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41). Isoamylase can split all the branching points of glycogen but not those of pullulan whereas pullulanase can split pullulan completely, but has limited hydrolytic activity on glycogen. Pullulan produced by *Aureobasidium pullulans* is a linear polymer of α-maltotriose joined endwise through (1,6-α) bonds. Isoamylase is particularly useful for elucidating the structures of glucans such as glycogen, whereas pullulanase was first used to clarify the structure of pullulan. There are several differences in the modes of decomposition of starch by isoamylase and pullulanase and these enzymes are useful for elucidation of the structure of starch. Isoamylase is produced in high yield by a mutant strain of *Pseudomonas amyloderamosa* SB15 and shows very high specific activity towards starch. This isoamylase is very useful for the industrial production of glucose or maltose from starch.

The major sugars produced from starch are glucose (dextrose), maltose, and

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isomerized sugar (isomerose). Isomerose is a mixture of glucose and fructose approaching the 50/50 composition of invert sugar, and which is increasingly being used as a substitute for sucrose, especially in soft drinks. (In acid drinks, sucrose is hydrolysed to invert sugar anyway, after a few days). Isomerose resembles invert sugar in being much sweeter than glucose and nearly as sweet as sucrose itself. There are now methods of obtaining pure fructose from isomerose but these are not dealt with in this review. The efficiency of production of isomerose from glucose depends very much on the purity of the glucose used as raw material: even a very small percentage of non-glucose sugars can have a disastrous effect on yields.

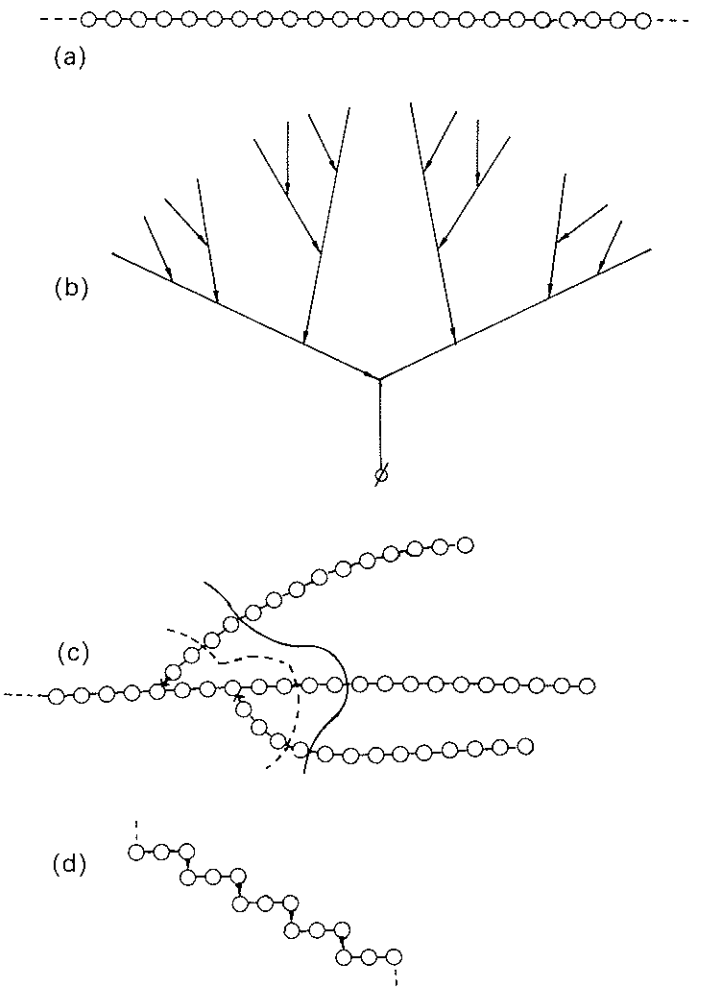

Figure 1. Structures of amylose, amylopectin and their related compounds. (1,4-α)-Linkages and (1,6-α)-linkages are represented by solid lines and by arrow heads, respectively. The position of the reducing end of the glucose unit is indicated by ∅. (a) Amylose; (b) Meyer's model of amylopectin and glycogen (this model is no longer recognized). The ratios of (1,6-α)-linkage to (1,4-α)-linkage are about 1 : 24 for amylopectin and about 1 : 12 for glycogen; (c) Amylopectin, amylopectin β-limit dextrin_____and phosphorylase_____limit dextrin; (d) Pullulan.

The major problems of conversion of starch into the desired sugars involve maximizing the yield of the target substance(s) with minimum contamination with undesirable by-products such as saccharides with (1,6-α)-links, retrograded starch and other higher polysaccharides. The reactions should be at the highest temperatures and reagent concentrations possible in order to economize in time, in enzymes and in the cost of removal of water in the preparation of stable syrups or pure solid products.

Structures of amylose, amylopectin and their related compounds are shown in *Figure 1*.

Occurrence of isoamylase

The name isoamylase was first given by Maruo and Kobayashi (1951) to an enzyme found in yeast cells and able to debranch intracellular glycogen. The enzymatic activity in these cells was too weak to allow detailed studies on the properties of the enzyme, which previously had been known as amylosynthetase. Lee, Nielsen and Fisher (1967) reported, however, that the combined actions of glucosyl transferase (4-α-D-glucanotransferase, EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) in an autolysate, debranch amylopectin to form a linear chain with a much longer chain length than the inner chain stubs of the original amylopectin. In 1968, Harada, Yokobayashi and Misaki selectively isolated colonies of *Pseudomonas* which develop a blue coloration with iodo-reagent in a medium containing amylopectin as the sole carbon source. The organism produced extracellular isoamylase and was named *Pseudomonas amyloderamosa* SB15. After this finding, many workers tried to obtain useful micro-organisms capable of producing isoamylase. In 1970, Gunja-Smith, Marshall and Whelan (1970) found the enzyme in a crude preparation of *Cytophaga*. Evans, Manners and Stark (1979) partially purified the enzyme and studied its properties, and Whelan's group (Gunja-Smith *et al.*, 1970; Marshall and Whelan, 1974) and Manners's group (Manners and Matheson, 1981) used the enzyme to examine the structures of glycogen and starch, respectively. Urlaub and Wöber (1975) detected isoamylase activity in an enzyme preparation without α-amylase extracted from cells of *Bacillus amyloliquefaciens* which produces α-amylase. Harada and Yokobayashi (unpublished data) could not detect the enzyme activity in the culture broth of 42 stock cultures of *Pseudomonas* tested in the Institute of Fermentation, Osaka: no strain other than the one that we had isolated was of industrial value, because other strains produced much lower amounts of enzyme.

Harada, Amemura and Konishi (unpublished data) showed that antiserum to *Pseudomonas* isoamylase formed a precipitin line with *Pseudomonas* isoamylase, but not with *Cytophaga* isoamylase. Thus, the two enzymes differ immunologically.

Occurrence of pullulanase

In 1961, pullulanase was discovered in *Aerobacter aerogenes* by Bender and Wallenfels (1961). Later, Walker (1968) detected pullulanase activity in cells of

some strains of *Streptococcus mitis* and (1,6-α)-glucosidase activity in place of pullulanase in three other strains of this species. The former strains did not accumulate glycogen, but the latter accumulated glycogen in the cells. A strain of *Streptomyces* (No. 280) was also reported to produce pullulanase (Yagisawa *et al.*, 1972). Griffin and Forgarty (1973) found a strain of *Bacillus polymyxa* that was able to produce pullulanase and β-amylase. A strain of *Bacillus cereus* var. *mycoides* was also shown to produce pullulanase and β-amylase extracellularly (Takasaki, 1976a). This enzyme preparation was reported to be useful for the production of maltose from starch (Takasaki, 1976b); Yamanobe and Takasaki, 1979). Morgan, Adams and Priest (1979) detected the enzyme activity in many strains of *Bacillus*. Recently, Novo Company (Norman, 1982a) found a strain of *Bacillus* capable of producing an acidic and thermophilic pullulanase and attempted to use it in conjunction with glucoamylase from *Aspergillus niger* for the production of glucose from starch. Wöber (1973) showed that *Pseudomonas stutzeri* produced pullulanase extracellularly.

Strains of *Aerobacter aerogenes* are classified as strains of *Klebsiella pneumoniae* and of *Enterobacter aerogenes*. Pullulanase was reported to occur in a strain of *Escherichia coli* (Palmer, Wöber and Whelan, 1973). Thus we (Konishi *et al.*, 1979) investigated the distribution of pullulanase in the family Enterobacteriaceae. First, we examined the growth of the organisms on a medium containing starch or pullulan, and their ability to produce pullulanase, finding that only species of *Klebsiella pneumoniae* can grow on such media and produce pullulanase: nine strains of *Enterobacter aerogenes* tested were unable to produce pullulanase. The presence of pullulanase was also examined by immunodiffusion. Culture filtrates and cell-free extracts of all eight strains of *Klebsiella pneumoniae* reacted immunologically with antiserum prepared with crystalline pullulanase from *Klebsiella pneumoniae* IFO 3321. Their precipitation lines fused completely with the homologous lines, except in the cases of *Klebsiella pneumoniae* IFO 3317 and ATCC 21073, the precipitation lines of which fused completely with each other, however. In contrast, nine strains of *Enterobacter aerogenes* did not cross-react with the antiserum. Thus, pullulanase formation may be a character common to all *Klebsiella* and, if so, it may be a key character in differentiating strains of *Klebsiella* from other strains of Enterobacteriaceae.

Palmer, Wöber and Whelan (1973) suggested that *E. coli* uses pullulanase for debranching of extracellular carbohydrate, whereas isoamylase is presumed to function intracellularly in the degradation of glycogen. Dessein and Schwartz (1974) reported, however, that no pullulanase activity was detectable in 11 different strains of *E. coli* grown on maltose and that neither these strains, nor 40 other strains of the same species, would grow on pullulan. We also could not detect pullulanase activity in cultures of two strains of *E. coli* tested. On the other hand, Jeannigros *et al.* (1976) detected activity of a debranching enzyme in a strain of *E. coli*. The purified enzyme hydrolysed (1,6-α)-glucosidic linkages in phosphorylase and β-amylase limit dextrins prepared from glycogen and amylopectin. It also completely hydrolysed amylopectin, but showed only very low activity on glycogen and no activity on pullulan. Thus, this enzyme cannot be classified as pullulanase or an isoamylase. Pullulanase has been shown to be produced by strains from *Nocardia*, *Lactobacillus*, *Micrococcus* and other

bacteria (Sakai, 1981). Pullulanase appears to be widely distributed in micro-organisms, in contrast to the limited distribution of isoamylase.

High activity of *Pseudomonas* isoamylase towards amylopectin and glycogen

The specific activities on starch and glycogen of our *Pseudomonas* isoamylase may be the highest reported for debranching enzymes. The debranching actions of crystalline isoamylase of *Pseudomonas* on amylopectin and glycogen have been investigated by Harada *et al.* (1972). The increase in reducing power was monitored and digests were fractionated on a Sephadex column. The results were compared with those obtained with crystalline pullulanase from *Klebsiella* (*Figure 2*). On incubation, 0·01 mg of the isoamylase hydrolysed the branching

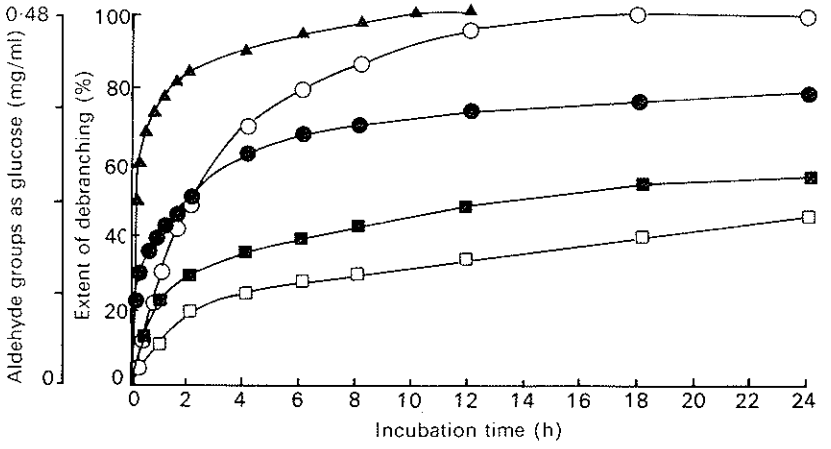

Figure 2. Debranching actions of purified enzymes on waxy-maize amylopectin (Harada *et al.*, 1972). One gram of amylopectin was treated with 0·001 mg (□) or 0·01 mg (○) of isoamylase, pH 3·5 or with 0·13 mg (■), 1·3 mg (●) or 13 mg (▲) of pullulanase, pH 5·5 in a volume of 100 ml.

linkages in 1 g of waxy-maize amylopectin in 20 h, whereas 1·3 mg of the pullulanase did not completely hydrolyse them even in 24 h, although 13 mg of the enzyme caused complete hydrolysis in 10 h. The distribution pattern of the linear (1,4-α)-linked unit chains in potato amylopectin is illustrated in *Figure 3* (Akai *et al.*, 1971b). Similar distribution patterns were obtained with waxy-maize amylopectin using *Pseudomonas* isoamylase and *Klebsiella* pullulanase (Lee, Mercier and Whelan, 1968), with waxy-rice amylopectin using *Pseudomonas* isoamylase (Akai *et al.*, 1971b) and with wheat amylopectin using *Cytophaga* isoamylase (Atwell, Hoseney and Lineback, 1980). There were two characteristic peaks with average degrees of polymerization (DP_n values) at the apex of about 50 and 20, respectively. Mercier and Kainuma (1975) showed that *Pseudomonas* isoamylase and *Klebsiella* pullulanase can be used in dimethylsulphoxide solution to study the fine structure of water-insoluble branched polysaccharides.

On incubation with oyster glycogen, 0·027 mg of the isoamylase caused com-

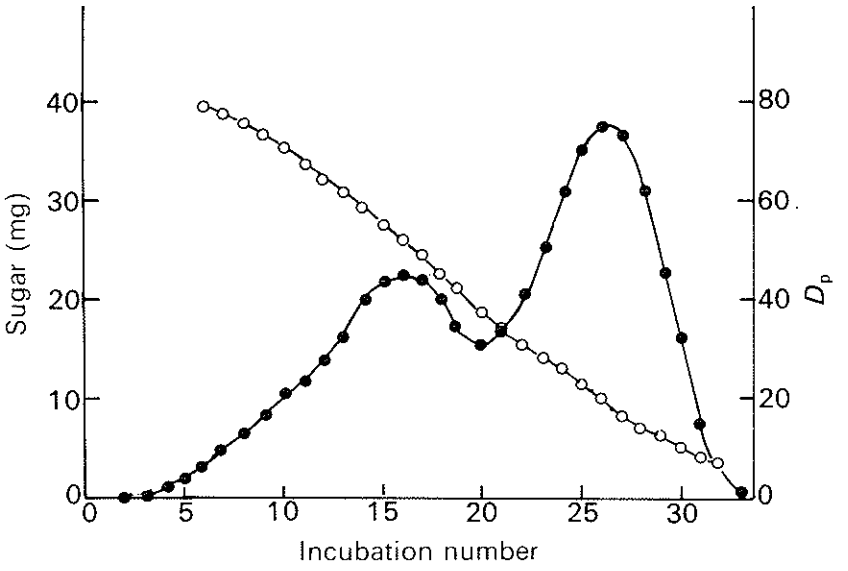

Figure 3. Fractionation of potato amylopectin (500 mg) on a Sephadex G-75 column after treatment for 20 h with *Pseudomonas* isoamylase (Akai *et al.*, 1971b). ○—○, D_p (degrees of polymerization); ●—●, mg sugar.

plete hydrolysis of the branching linkages in 24 h, while 29 mg of the pullulanase caused about 30% hydrolysis of the linkages in 24 h (Harada *et al.*, 1972). *Pseudomonas* isoamylase cleaved (1,6-α)-glucosidic linkages extensively to form linear (1,4-α)-linked unit chains of DP_n 15 from rabbit liver glycogen and DP_n 11 from oyster glycogen (Akai *et al.*, 1971a): the products gave one peak with a fairly broad distribution of chain lengths ranging from 3 to 50 glucose units; such patterns from glycogen were not obtained with pullulanase. *Pseudomonas* isoamylase has been used to clarify the structure of glycogens from various different sources, such as from the blue-green alga *Anacytis nidulans* (Weber and Wöber, 1975), and from shellfish such as scallops and abalone (Hata *et al.*, 1983) in addition to that from oysters (Akai *et al.*, 1971a; Umeki and Yamamoto, 1977).

The specific activities of *Pseudomonas* isoamylase were determined by Yokobayashi *et al.* (1972). The activities were measured as amounts of cleavage of glucosidic bonds per minute per milligram of protein at substrate concentrations of 2%, in comparison with those of *Klebsiella* pullulanase. The activities of *Pseudomonas* isoamylase were 110–280 μmol for amylopectins and glycogens and 1·1 μmol for pullulan, while those of *Klebsiella* pullulanase were 3–5 μmol for amylopectins, 0·5–1·2 μmol for glycogens and 53 μmol for pullulan. The K_m values (g/ml) of the isoamylase were about 1×10^{-4}–2×10^{-4} for amylopectins and glycogens and 2×10^{-3} for pullulan, whereas those of the pullulanase were about 8×10^{-3}–1×10^{-2} for amylopectins, about 2×10^{-2}–5×10^{-2} for glyco-

gens and $1{\cdot}7 \times 10^{-5}$ for pullulan. Thus, the debranching activities of isoamylase towards glycogen and amylopectin are much higher than those of pullulanase: the molecular activities of the isoamylase are about 9500–26 700 for amylopectins and glycogens, but only about 110 for pullulan whereas those of the pullulanase with amylopectin and glycogen were only 130–1400 and that with pullulan was 7700. These molecular activities were defined as the number of equivalents of a bond cleaved per minute per molecule of enzyme.

Comparison of substrate specificities of *Pseudomonas* isoamylase and *Klebsiella* pullulanase

The activities of *Pseudomonas* isoamylase and *Klebsiella* pullulanase for β-amylolysis of amylopectin, glycogen and related dextrins are shown in *Table 1* (Yokobayashi, Misaki and Harada, 1969, 1970). Isoamylase, like pullulanase, caused the complete breakdown of amylopectin, both when added before and also when added at the same time as β-amylase. Similarly isoamylase hydrolysed glycogen completely when acting either before or together with β-amylase. However, although pullulanase caused the complete degradation of glycogen when acting simultaneously with β-amylase, it caused only a slight increase in the β-amylolysis of glycogen when added before β-amylase. The incomplete degradation of glycogen by pullulanase is attributable to its inability to penetrate to the interior of the glycogen molecule. On the other hand, *Pseudomonas* isoamylase can penetrate the interior of the compact glycogen molecule and can hydrolyse all (1,6-α)-glucosidic inter-chain linkages. Isoamylase thus can be used to elucidate the structure of glycogen.

With glycogen β-limit dextrin, isoamylase caused extensive β-amylolysis (79%) when added before β-amylase, whereas pullulanase caused 31% degradation under the same conditions (*Table 1*), showing that isoamylase can split interior branch points in glycogen. Although the combined actions of *Pseudomonas* isoamylase and β-amylase caused degradation of β-limit dextrin, 20–30% of amylopectin β-limit dextrin and 20–25% of glycogen β-limit dextrin, respectively, were resistant to enzyme action. Pullulanase caused complete degradation of amylopectin β-limit dextrin both when added before and also when added at the same time as β-amylase; however, pullulanase caused only 32% degradation of glycogen when allowed to act before β-amylase.

The difference in the degradation rates of β-limit and phosphorylase limit dextrins by isoamylase can also be seen in *Table 1*. This difference is due to difference in the side chains in β-amylase limit dextrins and phosphorylase limit dextrins, the former being two or three glucose units long, and the latter four glucose units long (*see Figure 1c*). The action of pullulanase on waxy-maize amylopectin and oyster glycogen phosphorylase β-amylase limit dextrins, which have two glucose unit stubs, is similar to that on amylopectin, glycogen and their phosphorylase or β-amylase limit dextrin. However, the combined action of *Pseudomonas* isoamylase and β-amylase cause hydrolysis of 48% of amylopectin phosphorylase β-amylase limit dextrin and of 44% of the glycogen phosphorylase β-amylase limit dextrin. Gunja-Smith *et al.* (1970) noted in their report using *Cytophaga* isoamylase that 'if the ideal Meyer structure would be true,

Table 1. Effects of *Pseudomonas* isoamylase and *Klebsiella* pullulanase on β-amylolysis of amylopectin, glycogen and related dextrins (Yokobayashi, Misaki and Harada, 1969, 1970)

Substrate	Conversion to maltose (%)				
	β-Amylase alone	Action before β-amylase		Action simultaneous with that of β-amylase	
		Pseudomonas isoamylase	*Klebsiella* pullulanase	*Pseudomonas* isoamylase	*Klebsiella* pullulanase
Waxy-maize amylopectin	50	99	95	95	103
Potato amylopectin	47	96	98	97	103
Oyster glycogen	38	102	46	100	99
Rabbit liver glycogen	42	100	51	99	97
Waxy-maize amylopectin β-limit dextrin	0	80	97	72	97
Oyster glycogen β-limit dextrin	0	79	31	76	99
Waxy-maize amylopectin phosphorylase limit dextrin	21	95	97	98	101
Rabbit liver glycogen phosphorylase limit dextrin	28	94	32	97	99
Waxy-maize amylopectin phosphorylase β-limit dextrin	0	48	96	56	100
Rabbit liver glycogen phosphorylase β-limit dextrin	0	44	29	50	97

every B chain liberated by isoamylase from the Meyer phosphorylase β-amylase limit dextrin would be branched near its non-reducing end by a maltosyl A chain. Thus, the debranched molecule should remain inert to the action of β-amylase'. However, the extent of amylolysis of isoamylase-treated phosphorylase β-limit dextrin is significantly high, as shown in our experiments and theirs. There are several possible explanations for this. One possibility is that B chains do not carry an A chain. This was postulated by Gunja-Smith *et al.* (1970) who then revised the Meyer branched model of glycogen and amylopectin (the Meyer structure is no longer recognized). The second possibility is that there may be 'buried chains' in the structure: that is, that some of the A chains are not subject to the action of phosphorylase and β-amylase. There is a third possibility, that the preparations of phosphorylase β-limit dextrin used by Yokobayashi, Misaki and Harada (1970) and by Gunja-Smith *et al.* (1970) have branched points with maltotriose stubs as pointed out by Nakamura (1977). Another possibility is that isoamylase can split some of branching points with maltose stubs in them. We (Yokobayashi, Misaki and Harada, 1970) have shown that *Pseudomonas* isoamylase liberates maltose and maltotriose in a molar ratio of 1 : 13·4 with amylopectin β-limit dextrin and 1 : 9·7 with glycogen β-limit dextrin under our conditions. Evans, Manners and Stark (1979) also detected the presence of maltose in a reaction mixture containing a purified *Cytophaga* isoamylase and amylopectin. Maltose production was also clearly observed after a longer incubation by Manners and Matheson (1981). Isoamylase can slowly split branching points with maltose stubs in β-limit dextrin, and this may explain in part why phosphorylase β-limit dextrin with maltose stubs is degraded by the action of isoamylase. The comparatively poor ability of isoamylase to hydrolyse such branching points with maltose stubs does not affect its efficiency in the industrial production of sugars from starch.

The detailed structural requirements of the substrate of *Pseudomonas* isoamylase were compared qualitatively and quantitatively with those of *Klebsiella* pullulanase (Kainuma, Kobayashi and Harada, 1978) and some of the data are shown in *Figure 4* and *Table 2*. The best substrates for the isoamylase are polymers of higher molecular weight, such as amylopectin and glycogen, whereas those of the pullulanase are branched oligosaccharides derived from amylopectin. An important difference between isoamylase and pullulanase is that the former only slowly hydrolyses pullulan whereas pullulanase has a similarly slow action on glycogen. Maltosyl branches in the oligosaccharides are hydrolysed

Table 2. Relative reaction rates of *Pseudomonas* isoamylase and *Klebsiella* pullulanase on various branched oligosaccharides and polysaccharides (Kainuma, Kobayashi and Harada, 1978)

Substrate	*Pseudomonas* isoamylase	*Klebsiella* pullulanase
Potato amylopectin	100	15
Oyster glycogen	124	1
Pullulan	1	100
6^3-*O*-α-Maltosyl-maltotriose	3	22
6^3-*O*-α-Maltotriosyl-maltotriose	10	162
6^2-*O*-α-Maltotetraosyl-maltotriose	7	26
6^3-*O*-α-Maltotriosyl-maltotetraose	33	146

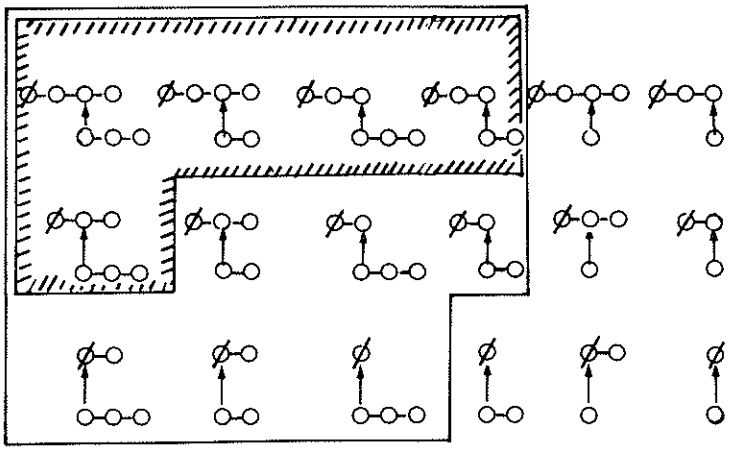

Figure 4. Susceptibility of branched oligosaccharides to *Pseudomonas* isoamylase and *Klebsiella* pullulanase in relation to their structures (Kainuma, Kobayashi and Harada, 1978). Boxed area susceptible to pullulanase and isoamylase. (1,4-α)-Linkages and (1,6-α)-linkages are represented by solid lines and by arrow heads, respectively. The position of the reducing end of the glucose unit is indicated by ∅.

by *Pseudomonas* isoamylase much more slowly than the maltotriosyl branches. *Pseudomonas* isoamylase requires a minimum of three D-glucose residues in the chains at the reducing end of the branched oligosaccharides.

Endo-type and exo-type cleavage of amylopectin and glycogen, respectively, by *Pseudomonas* isoamylase

In order to determine whether isoamylase has an endolytic or exolytic action on branching points with (1,6-α)-glucosidic linkages in amylopectin and glycogen, the hydrolysis products with isoamylase, obtained after appropriate periods, were fractionated on Sephadex G-75 and the sugars in each fraction were measured (Harada *et al.*, 1972), as shown in *Figures 5* and *6*. For comparison, the action of pullulanase was also examined. After about 50% or 70% hydrolysis, the products obtained with isoamylase clearly contained less residual polymer and more large glucans with branching linkages than the products of pullulanase action. Amounts of 0·01 mg of isoamylase or 1·3 mg of pullulanase caused about 50% hydrolysis of 1 g of waxy-maize amylopectin in 2 h. After 80–85% hydrolysis with isoamylase, the amounts of residual polymer had diminished and two peaks appeared, one peak being that of linear glucan and the other peak probably being that of linear glucans and glucans containing branching linkages. Isoamylase differs from pullulanase in that it can hydrolyse higher-molecular-weight branched glucans more readily than branched oligosaccharides of lower molecular weight. The reaction mixture became white due to precipitation at an early stage of the reaction with isoamylase. This precipitation could have been caused by the retrodegradation of the short-chain amylose and branched oligosaccharides produced. After 24 h incubation with isoamylase a typical pattern of amylopectin products with linear linkages was seen, but after 24 h incubation with pullulanase there was still a great deal of residual glucan. Incubation with

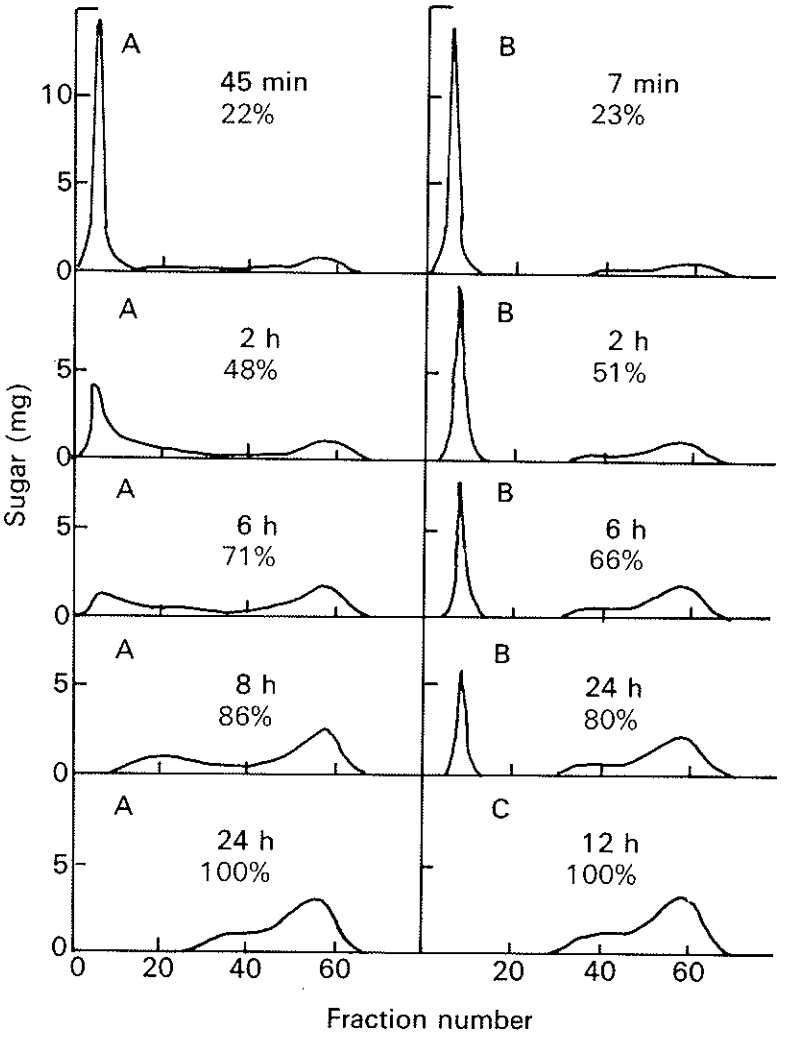

Figure 5. Fractionation of waxy-maize amylopectin on a Sephadex G-75 column after enzyme treatment (Harada *et al.*, 1972). A = 0·01 mg isoamylase; B = 1·3 mg pullulanase; C = 13 mg pullulanase. Incubation time and degree of hydrolysis is shown for each fractionation pattern.

a tenfold increase in pullulanase (13 mg) for 12 h resulted in complete scission of the branching linkages of amylopectin, giving the same pattern of linear linkages as that obtained with isoamylase. These observations suggest that the isoamylase hydrolyses both inner and outer branching linkages of amylopectin, whereas the pullulanase hydrolyses outer linkages of amylopectin efficiently, but affects inner linkages only slowly.

Significant differences between the actions of the two enzymes on glycogen were observed: the isoamylase hydrolysed all the branching linkages comparatively well, whereas the pullulanase hydrolysed relatively few. The isoamylase

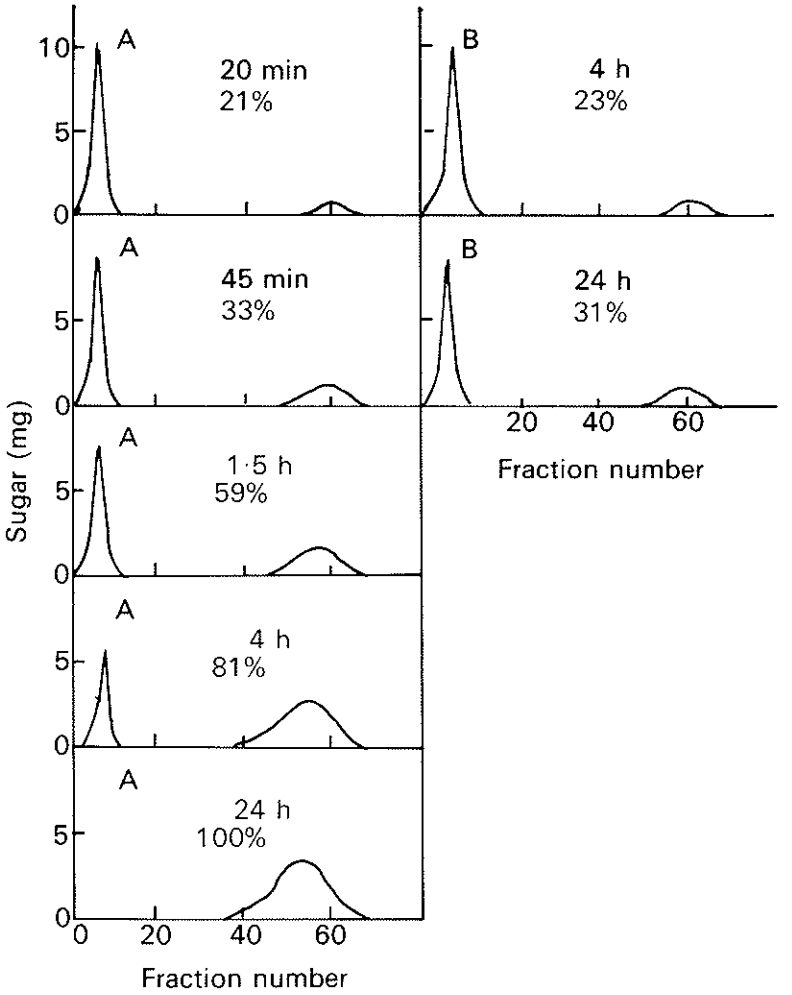

Figure 6. Fractionation of oyster glycogen on a Sephadex G-75 column after enzyme treatment (Harada *et al.*, 1972). A = 0·027 mg isoamylase; B = 29 mg pullulanase. Incubation time and degree of hydrolysis shown for each fractionation pattern.

hydrolysed glycogen exolytically: this action is attributable to the structure of the polysaccharide, not to the specificity of binding of the active site of the enzyme. Palmer, Macakie and Grewal (1983) extended these experiments using *Cytophaga* isoamylase and suggested that, during hydrolysis by the enzyme, branched oligosaccharides are not produced in measurable amounts from glycogen. These findings suggest that isoamylase from *Pseudomonas* and *Cytophaga* have exolytic actions on glycogen; i.e. they catalyse the ordered and sequential hydrolysis of (1,6-α)-glucosidic linkages proceeding from the non-reducing ends to the interior of the chain.

Enhanced production of isoamylase by *Pseudomonas amyloderamosa*

Pseudomonas amyloderamosa SB15 produces isoamylase extracellularly, and no other amylase or α-glycosidase apart from isoamylase can be detected in the culture filtrate of this organism, thus providing a very convenient method of obtaining pure isoamylase, whereas contaminating enzymes usually impede enzyme purification.

We have used an iodine colour test to detect and isolate organisms that produce only isoamylase. In the test, filter paper is dipped in a 1% solution of waxy amylopectin containing 0·1 M acetate buffer, pH 3·5, and the wet paper is placed on the colonies formed on agar medium and incubated at 40°C for 30 min. The paper is then exposed to iodine vapour: blue spots indicate the position of colonies that produce isoamylase constitutively. These colonies were picked up by the replica method.

Pseudomonas amyloderamosa SB15 produces isoamylase inducibly (i.e. it can be induced to do so) in the presence of glycogen, starch or maltose. The most satisfactory medium consists of 2% maltose or starch, 0·4% sodium glutamate, 0·3% diammonium hydrogen phosphate, and other inorganic salts. This organism requires glutamate or aspartate for growth. The optimum pH for enzyme production is 5–6; the enzyme is stable at pH 2–6, but is extremely labile above pH 7. The maximum yield of the enzyme is obtained after 2 days in the above culture medium (Harada, Yokobayashi and Misaki, 1968).

We used mutation procedures to increase the enzyme production by this organism over 500-fold. Sugimoto, Amemura and Harada (1974), using the test paper method (Harada, 1965), by the following procedure, isolated a constitutive mutant strain MS1 that produces isoamylase: strain SB15 cells treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, were examined by the iodine colour test paper method already described; we then selected much better producers from the original strain by the iodine test paper method, and finally obtained the best mutant strain 1168, which is now used industrially.

Short-chain amyloses formed by the action of extracellular isoamylase can enter the cells in which amyloses are degraded by α-amylase (Kato *et al.*, 1975) and α-glucosidase (Amemura, Sugimoto and Harada, 1974) to form glucose as a final product. The α-amylase attacks amylose much faster than it does amylopectin and glycogen: this enzyme may therefore have a role in degrading short-chain amylose entering the cells after formation by the action of extracellular isoamylase. This α-amylase produces almost equimolar amounts of glucose and maltose as final products and the resultant maltose is hydrolysed by the action of the α-glucosidase. The parent strain SB15 can produce the two intracellular enzymes inducibly, as well as isoamylase, but the mutant strain MS1 produces them constitutively (i.e. as a normal process). The extracellular isoamylase and two intracellular enzymes together permit utilization of extracellular branched α-glucans such as glycogen and amylopectin.

An isoamylase-negative mutant strain, K1, was derived from MS1, and K1C was obtained as a revertant of K1 (Kuswanto *et al.*, 1976). Strain K1 grew as poorly on glycogen or amylopectin medium as on a non-carbohydrate medium. It has no isoamylase activity, and α-amylase and α-glucosidase activities similar

to those of the parent strain MS1. Norrman and Wöber (1975) reported that strain SB15 showed a preference for glycogen to amylopectin for growth, but we have not been able to confirm this.

Isoamylase can easily be purified with cross-linked amylose gel as an absorbent (Kato *et al.*, 1977). The enzyme can be eluted from the gel with 5% maltotriose or linear maltodextrin solution. Crystalline enzyme was prepared by dropwise addition of ammonium sulphate to a solution of the purified enzyme (*Figure 7*). By this procedure 96 mg of the enzyme was purified to homogeneity from 20 litres of culture broth of strain K1C with a yield of about 70%. Strain 1168 produces about 500 mg of isoamylase as the purified enzyme per litre of culture broth.

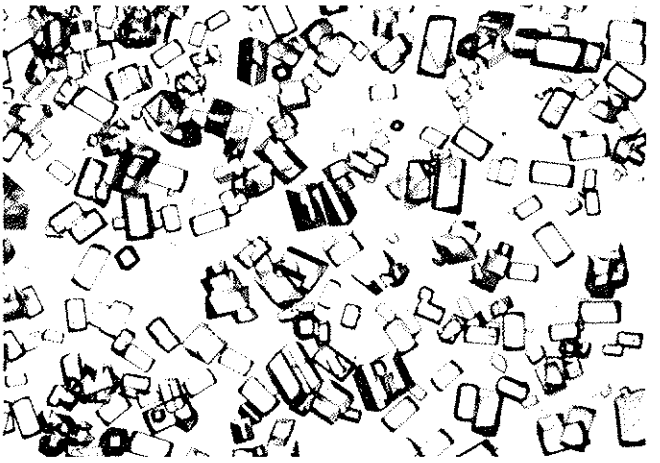

Figure 7. Photograph of crystalline isoamylase from *Pseudomonas amyloderamosa* SB15.

Molecular properties of *Pseudomonas* isoamylase

Crystalline isoamylase (*Figure 7*) was found to be contaminated with a trace of proteolytic enzyme (Amemura, Konishi and Harada, 1980). This contaminant digested the isoamylase under neutral or alkaline conditions, especially in the presence of sodium dodecyl sulphate. A reliable molecular weight of the enzyme was obtained by SDS-polyacrylamide gel electrophoresis and by gel filtration on Sepharose-6B in 6 M guanidine hydrochloride after heat inactivation of the proteolytic contaminant. The molecular weight of the undegraded polypeptide chain of the isoamylase was about 90 000: that of *Klebsiella* pullulanase is 143 000 (Eisele, Rashed and Wallenfels, 1972). Comparison of the amino acid compositions of isoamylase and pullulanase has shown that isoamylase has a much lower lysine content (Kitagawa, Amemura and Harada, 1975). Isoamylase has no sugar component, but the pullulanase has a sugar content of about 2%. The effects of inhibitors on the two enzymes were compared: an SH group did not seem to be related to the activity of either enzyme, but isoamylase is inhibited by iodo-2,4-nitrofluorobenzene and Hg ion. These results and the pK_a value suggest that an imidazole radical may be important for the activity of isoamylase.

Tryptophan groups may not be essential for the activity of isoamylase, although, like pullulanase, the enzyme was inhibited by *N*-bromosuccinimide and 2-hydroxy-5-nitro-benzylbromide. A tryptophan group may be important for the activity of pullulanase (Amemura, Kitagawa and Harada, 1975). Isoamylase was inhibited by maltotriose but not by maltose, which would explain why it does not attack 6-α-maltosyl maltodextrin. The fact that maltose is not inhibitory is very useful in the production of maltose by the combined actions of isoamylase and β-amylase, as shown later. Isoamylase was not inhibited by cyclodextrin, whereas pullulanase was (Marshall, 1973). Isoamylase was inactivated more than pululanase by photo-oxidation in the presence of 0·001% rose bengal (Kato and Harada, unpublished).

Preliminary X-ray studies were carried out on isoamylase in the crystalline state and in aqueous solution (Sato *et al.*, 1982). The diffraction patterns of the h0l and 0kl zones were recorded with a Buerger precession camera. The crystal is orthorhombic, the space group is $p2_12_12_1$, and the unit-cell dimensions are $a = 137{\cdot}9$, $b = 52{\cdot}9$, $c = 151{\cdot}2$ Å ($V = 1{\cdot}1 \times 10^6$ Å^3). If we assume that the asymmetric unit contains one enzyme molecule of molecular weight 95 000, the V_m and V_{prot} values are within the range usually found for proteins. Solutions at concentrations of 0·4, 0·8, 1·0 and 1·5% were prepared for the X-ray generator. Guinier plots for these four solutions showed that the size and shape of the enzyme molecule are uniform. The four plots were essentially parallel.

The radii of gyration of this enzyme molecule, obtained from these plots, showed a good coincidence, giving an R_g value of 27·5 Å (2·75 nm) after slit error correction. The approximate maximum dimension of the enzyme can be obtained by Fourier transformation of the small-angle scattering intensity observed, the so-called $p(r)$ function, 84 Å (8.4 nm). Referring to the results obtained for Takaamylase A (from *Aspergillus oryzae*) (molecular weight 45 000), we finally concluded that the molecule is prolate.

Glucoamylase, glucose isomerase and β-amylase; the principal enzymes for production of glucose, isomerized sugar and maltose

In 1958, Tsujisaka, Fukumoto and Yamamoto developed a method for the commercial production of glucose using an enzyme from a strain of *Rhizopus delemar*. Subsequently, good producers of glucoamylase, such as strains of *Aspergillus niger* have been discovered (Underkofler, Denault and Hou, 1965; Freedberg *et al.*, 1974). The enzyme from *Aspergillus niger* has been used for the production of glucose from starch in many countries. In Japan the enzymes from *Rhizopus delemar* or *Rhizopus niveus* also have been used industrially. A strain of *Endomycopsis fibuligera* (old name, *Endomyces fibuliger*) (Hattori and Takeuchi, 1961) was used in some Japanese companies at an early stage of the industry. The optimum pH and temperature of enzymes from *Aspergillus niger* are 4·5 and 60°C, whereas those from *Rhizopus* are 5·0 and 56°C. Enzymes from *Aspergillus niger* can be produced by liquid culture, but enzymes from *Rhizopus* are produced by culture on solids. Thus, in general, the former enzyme is preferred to the latter enzyme for industrial purposes. *Rhizopus* sp. (Ueda, Ohba and Kano, 1975) and *Aspergillus niger* (Smiley *et al.*, 1971) have multiple gluco-

amylases, whereas *Endomycopsis fibuligera* has a single enzyme (Fukui and Nikuni, 1969; Sukhumavasi, Kato and Harada, 1975; Kato *et al.*, 1976). A strain of *Rhizopus* produces glucoamylases I and II; the former, which is capable of hydrolysing raw starch, can hydrolyse the (1,6-α)-glucosidic linkage, but the latter cannot (Ueda, 1980). Many strains of *Rhizopus*, *Aspergillus niger* and *Endomycopsis fibuligera* have been used to produce the traditional fermented foods in South-East Asia: *Rhizopus* is used for Chinese liquor in China and for Tempe in Indonesia, *Aspergillus niger* for the special Japanese beverage Shochu, and *Endomycopsis fibuligera* for the production of sugar syrups from starch—in Thailand as Look Pang, in Indonesia as Ragi, and in the Philippines as Busbad. The glucoamylases from *Aspergillus awamori* (Ueda, 1957; Ueda, Ohba and Kano, 1974; Gasdorf *et al.*, 1975; Hayashida *et al.*, 1982) and *Aspergillus oryzae* (Morita *et al.*, 1966; Miah and Ueda, 1977a, b) which have been used for the production of the Japanese beverages Awamori and Sake, respectively, also consist of two or three forms. *Penicillium oxalicum* also has two forms (Yamasaki, Suzuki and Ozawa, 1977). The glucoamylase preparation of *Aspergillus niger*, Glucozyme XL-128 (Nagase Co.) contains a phosphatase (EC 3.1.3.1), the concomitant action of which was shown by Abe, Takeda and Hizukuri (1982) to be necessary for the complete hydrolysis of the starch. These workers mentioned that the idea that glucoamylases of various origins are capable of the complete hydrolysis of starch should be reconsidered, in the light of the presence of phosphatase, because starch can bind considerable amounts of phosphate covalently.

Glucoamylase can polymerize glucose by a reaction that is the reverse of hydrolysis (Fukumoto, 1969; Hehre, Okada and Genghof, 1969). The main products of this reverse reaction are maltose and isomaltose, although on prolonged incubation at high substrate concentrations other disaccharides can be detected. Polymerization can also occur by another mechanism, namely glucose transfer, which is catalysed by α-glucosidase (EC 3.2.1.20); this reaction was also shown to be catalysed by a transglucosylase (Pazur and Ando, 1961), which is often present as an impurity in crude glucoamylase preparations (Maher, 1968). Currently used commercial preparations of glucoamylase of many different origins do not now contain glucose transferase activity: Novo Company isolated a mutant strain of *Aspergillus niger* which was devoid of such transferase activity. Most α-glucosidase catalyses the formation of non-fermentable glucose oligomers by transferring a glucosyl moiety from the (1,4-α) to a (1,6-α) position. If transferase activity is present, considerable amounts of pannose and isomaltose are formed during saccharification and so the final yield of glucose is considerably reduced.

In 1957, Marshall and Kooi found that *Pseudomonas hydrophila* accumulates glucose isomerase in the cells when grown on xylose. Subsequently, numerous producers of the enzyme have been discovered by many research workers. In 1964 and 1965, D-xylose ketol-isomerase (xylose isomerase, EC 5.3.1.5) was discovered in strains of *Streptomyces* independently by two groups in Japan (Takasaki and Tanabe, 1964; Sato and Tsumura, 1964; Tsumura and Sato, 1965; Takasaki, 1966). This enzyme is called expediently glucose isomerase: it can convert glucose to fructose efficiently at a temperature of about 70°C. Thus, this enzyme was

developed for commercial production of isomerized sugar composed of about equal amounts of glucose and fructose. This enzyme is induced by xylose, but Takasaki, Kosugi and Kanbayashi (1969) isolated a mutant strain of *Streptomyces albus* capable of using xylan as a much less expensive inducer than xylose to produce the enzyme. Later, Sanchez and Quinto (1975) isolated a D-xylose isomerase constitutive mutant strain that was insensitive to D-glucose repression, and Novo Company found a strain of *Bacillus coagulans* capable of producing glucose isomerase and developed the enzyme on an industrial scale.

The isomerase is immobilized by a chemical process in which the enzyme is bound to a solid support. Many companies such as Denki Kagaku Company and Novo Company have developed immobilized glucose isomerase to make isomerized sugar. Isomerized sugar being a mixture of glucose and fructose, comparable with invert sugar, is used in large amounts in food processing, particularly in soft-drink bottling; for this purpose it is used as a substitute for sucrose. In Japan about 500 000 t of isomerized sugar currently are produced commercially (*see* Chapter 5).

β-Amylase (EC 3.2.1.2) occurs in higher plants, such as sweet potato, soya bean, wheat, barley, oats, maize and rye. Robyt and French (1964) found an enzyme producing maltose in the culture broth of *Bacillus polymyxa* and later Higashihara and Okada (1974) discovered β-amylase in *Bacillus megatherium*. β-Amylase was also found in many strains of bacteria, such as *Bacillus* sp. (Shinke, Kunimi and Nishira, 1975b), *Streptomyces* sp. (Shinke, Nishira and Mugibayashi, 1974) and *Pseudomonas* sp. (Shinke, Kunimi and Nishira, 1975a). Some of these strains produce β-amylase with pullulanase. However, crude enzyme from soya bean has been used for commercial production of maltose in Japan, because much soya bean protein is produced industrially in Japan and crude β-amylase can be obtained fairly easily from it. The properties of soya bean β-amylase have been studied by Mikami, Aibara and Morita (1982).

Use of isoamylase in glucose production from starch

Glucose syrups are used for the production of glucose and as the starting material for the production of isomerized sugar consisting of equal amounts of glucose and fructose. Such syrups are mainly produced from starch by the action of glucoamylase, instead of the previously used (and now discontinued) acid treatment, which gave a lower glucose yield and an undesirable colour, derived from the sugar. Glucoamylase can hydrolyse (1,6-α)-glucosidic linkages, but at a much slower rate than (1,4-α)-glucosidic linkages. Industrially, it is desirable to obtain syrups with a high glucose content from starch: this can be achieved using a debranching enzyme with glucoamylase for the saccharification of starch (Harada *et al.*, 1982; Norman, 1982a, b).

One example of our experiments on the possible commercial production of glucose from starch was as follows. A 33% suspension of corn starch was liquefied by the action of Termamyl, a thermostable α-amylase (Madsen, Norman and Scott, 1973) at 98°C for 30 min. The optimum pH of the isoamylase is 3·0–4·5 at 30°C and about 4·0–4·5 at 55°C, whereas that of glucoamylase is 4·0–4·5. A pH of 4·5 was employed for the combined actions of glucoamylase from *Asper-*

gillus niger (amyloglucosidase Novo 150) and *Pseudomonas* isoamylase. The optimum temperature for the action of isoamylase is 50–55°C. Norman (1982b) reported that the limiting temperature for practical application of the glucoamylase is about 60°C. We examined the effect of temperatures of 50°C, 55°C and 60°C on the yield of glucose and found that 55°C was by far the best. The yield of glucose on hydrolysis at 55°C for 40 h was 95·9% whereas it was 94·2% when only glucoamylase was used.

Norman (1982a, b) examined the use of a debranching enzyme, *Pseudomonas* isoamylase or *Bacillus* pullulanase, in glucose syrup production and showed that only the *Bacillus* pullulanase can act at 60°C, a temperature which is useful for industrial operation because it prevents microbial contamination during saccharification. However, *Pseudomonas* isoamylase is much more acidophilic than *Bacillus* pullulanase and *Klebsiella* pullulanase, the optimum pH of *Bacillus* pullulanase being 4·5–5·5 whereas that of *Klebsiella* pullulanase is 5·5–6·0. Combined activity of glucoamylase and *Bacillus* pullulanase can be achieved at pH 4·5–5·0, whereas that of glucoamylase and isoamylase is achieved at pH 4·0–4·5. *Klebsiella* pullulanase is unsuitable for such combined activity because its optimum pH is much higher than that of glucoamylase. Thus, a much lower pH can be used for isoamylase than for *Bacillus* pullulanase. Use of the isoamylase which is capable of acting at such a low pH is advantageous for avoiding microbial contamination. If isoamylase or pullulanase is used with glucoamylase, the amount of glucoamylase required to obtain a high level of glucose can be reduced: the amount of glucoamylase required decreases as the amount of added isoamylase is increased. Glucoamylase reversion reaction products can thus be reduced, leading to an increase of glucose yield. Use of debranching enzyme and the glucoamylase system not only leads to a higher glucose level but also to a much shorter incubation time. The yield of glucose increases greatly with increase in the concentration of isoamylase used.

The ability of a particular glucoamylase to digest raw starch depends not only on the amylase activity, but also on the debranching activity of the enzymes. *Pseudomonas* isoamylase effectively enhances the production of glucose from raw starch by glucoamylase, as shown by Ueda, Ohba and Kano (1974). This process is important in the production of ethanol from starch.

Use of isoamylase in maltose production from starch

The action of β-amylase alone on amylopectin results in the formation of maltose (50–60% yield), leaving β-limit dextrin. On addition of isoamylase or pullulanase, the branching point of amylopectin is cleaved and the yield of maltose greatly increases. The following experiment was used to assess the commercial production of maltose from starch with *Pseudomonas* isoamylase and soya bean β-amylase (Harada *et al.*, 1982). An aqueous suspension of 20% corn starch was liquefied by the action of Termamyl and was then treated simultaneously (at various pH values of 3·5–6·0 at 50°C for 48 h) with β-amylase from soya bean and with *Pseudomonas* isoamylase. Bacterial α-amylase, which can cleave maltotriose to form maltose and glucose, was added to the reaction mixture to enhance maltose production. A pH of 4·5 was best for maltose production: this is under-

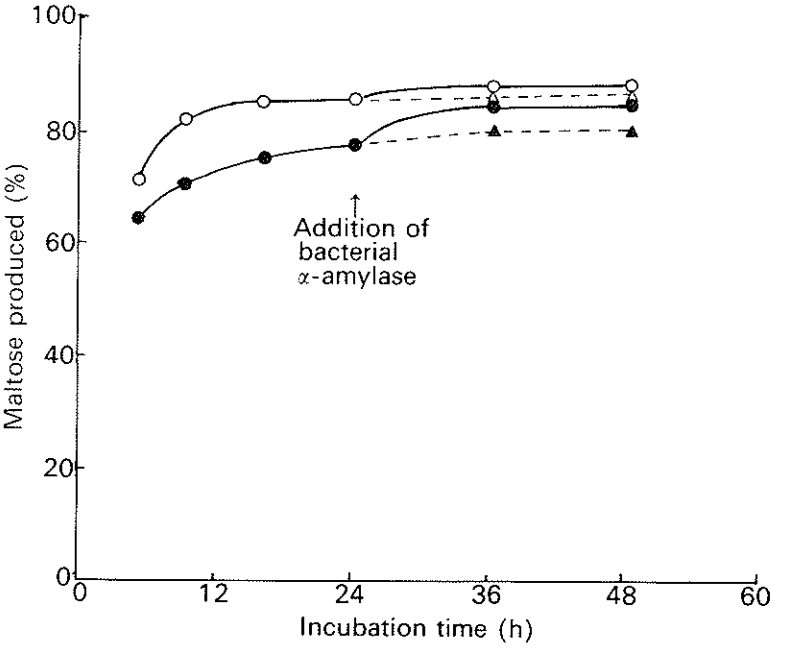

Figure 8. Production of maltose from corn starch at concentration of 21% (DE, 2·9) at 50°C by soya bean β-amylase (20 μg/g starch) with isoamylase (○) (6 μg/g starch) at pH 4·7 or pullulanase (●) (17 μg/g starch) at pH 6·0 (Harada *et al.*, 1982). The amounts of the enzyme used were expressed as those of purified enzyme. DE (dextrose equivalent), reducing sugars expressed as D-glucose and calculated as a % of dry substance. The curves with isoamylase (△) or pullulanase (▲) with β-amylase without bacterial α-amylase are shown as broken lines.

standable, bearing in mind that the optimum pH of the β-amylase is 5·0–6·0 and that of the isoamylase is 4·0–4·5 at 55°C. *Pseudomonas* isoamylase was compared with *Klebsiella* pullulanase (*Figure 8*). A pH of 4·7 was used for the isoamylase, but pullulanase was tested at pH 6·0 because the optimum pH of pullulanase is 5·5–6·0. The yield of maltose is higher with *Pseudomonas* isoamylase (87·6%) than with *Klebsiella* pullulanase (85·6%). In these experiments three times more pullulanase than isoamylase was added. High concentrations of substrate, such as the 20% used in this experiment, give a much lower maltose yield than do low concentrations such as 0·1% (*Table 1*). Chain-stubs of starch are composed of odd or even numbers of glucose residues: small amounts of glucose and maltotriose therefore are produced with maltose from starch by the simultaneous actions of β-amylase and isoamylase. To enhance the yield of maltose, the development of a precipitate due to retrogradation of the products formed by isoamylase should be minimized, by adding isoamylase at the most appropriate time and by adding isoamylase and glucoamylase or β-amylase in the correct proportions.

Some maltose produced by the Hayashibara Company (over 1000 t/year) is sold by the Ootsuka Pharmaceutical Company under the trade name of Martos 10. Maltose may be preferred to glucose for intravenous injection because of its lower osmotic pressure and slower release of glucose so that the time required

for injection of glucose may be shortened when maltose is injected at an equivalent level to glucose. The Hayashibara Company succeeded in producing crystalline maltose and now produces over twenty thousand tons per year as a food sweetener. Maltose is a much better sweetener than sucrose for use in Japanese confectionery because it does not easily become crystalline when kept for a long time, whereas sucrose readily crystallizes in confectionery. This company has also succeeded in making crystalline maltitol, which can easily be obtained from maltose by chemical reduction; Ohno, Hirao and Kido (1982) have studied it by X-ray diffraction methods. Purified maltitol is not hygroscopic and therefore has numerous applications. In co-operation with other companies, Hayashibara Company produces now over 3000 t of maltitol per year as a low-calorie sweetener. Atsuji *et al.* (1972) and Oku, Him and Hosoya (1981) reported that maltitol is a low-calorie sweetener and Rennhard and Bianchine (1976) reported that maltitol is readily metabolized in rat, dog and man.

Maltose is almost as sweet as sucrose (Oda, 1974). Japan has the greatest commercial production of maltose and *Pseudomonas* isoamylase and soya bean β-amylase are mainly used for its production. *Pseudomonas* isoamylase is preferable to *Klebsiella* pullulanase for this purpose for the following reasons: (1) isoamylase splits over 20 times more (1,6-α)-glucosidic linkages in amylopectin than pullulanase per unit of enzyme protein; (2) because the action of isoamylase is not inhibited by maltose, a higher concentration of starch can be used as starting material; (3) the reaction of the isoamylase is non-reversible, whereas that of pullulanase is reversible. Pullulanase polymerizes maltose, forming a tetrasaccharide, and links maltose with amylose (Abdullah and French, 1970); the yield of maltose is therefore much higher with isoamylase than with pullulanase.

An account of the uses of debranching enzymes in the brewing industry has been given by Enevoldsen (1975).

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