Purification and characterization of an alkaline isoamylase from an alkalophilic strain of *Bacillus*

KATSUTOSHI ARA,* KATSUHISA SAEKI and SUSUMU ITO

Tochigi Research Laboratories of Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-34, Japan

(Received 14 September 1992; revised 25 November 1992; accepted 9 December 1992)

Alkaline isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) activity was detected in the culture medium of an alkalophilic strain of *Bacillus* sp., designated KSM-3309, which was isolated from a soil sample. This novel enzyme was purified to homogeneity from the culture filtrate by precipitation with ammonium sulphate, chromatography on DEAE-cellulose and DEAE-Bio-Gel A, and gel filtration on Sephacryl S-200. The purified enzyme had a pH optimum of approximately 9.0, and displayed maximum catalytic activity at 55 °C. The enzyme had a molecular mass of 65 kDa, as determined by both SDS-polyacrylamide gel electrophoresis and gel filtration on Sephacryl S-200. The isoelectric point was 4.2. This enzyme cleaved the branching points of both amylopectin and glycogen, and incubation of the enzyme with these glucans caused large increases in coloration of the iodine reagent. Amylose, pullulan and maltose were practically insensitive to the enzyme. The enzyme activity was inhibited by Hg²⁺ ions and by *N*-bromosuccinimide, but the thiol inhibitors iodoacetate, 4-chloromercuribenzoate and *N*-ethylmaleimide had either no effect or a slightly inhibitory effect. β -Cyclodextrin, an inhibitor of pullulanase, was not inhibitory.

Introduction

Unlike α -amylase (1,4- α -D-glucan 4-glucanohydrolase, EC 3.2.1.1), isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68) and pullulanase (pullulan 6-glucanohydrolase, EC 3.2.1.41) hydrolyse the 1-6- α -D-glucosidic inter-chain linkages of certain branched α -D-glucans. The enzymes differ mainly in that isoamylase cannot hydrolyse pullulan. Isoamylase was first found in autolysed brewers' yeast (Maruo & Kobayashi, 1951) and then in bakers' yeast (Gunja *et al.*, 1961). Harada *et al.* (1968) identified an extracellular isoamylase produced by *Pseudomonas amyloclavata*, which was then purified and characterized (Yokobayashi *et al.*, 1970; Harada *et al.*, 1972). Subsequently, several bacteria, such as *Cytophaga* sp. (Gunja-Smith *et al.*, 1970a), *Bacillus amyloliquefaciens* (Urlaub & Wober, 1975), *Escherichia coli* (Jeanningros *et al.*, 1975), *Flavobacterium* sp. (Sato & Park, 1980), and the yeast *Lipomyces kononenkoae* (Spencer-Martins, 1982) were found to produce isoamylases. These isoamylases, including pullulanases, can potentially be used in the elucidation of the fine structures of polysaccharides and related α -glucans (Gunja-Smith *et al.*, 1970b; Akai *et al.*, 1971; Hizukuri *et al.*, 1983) and

for the industrial production of amylose, maltose and glucose from starch, in combination with other amylolytic enzymes (Norman, 1982; Stominska & Maczynski, 1985).

Recently, we have found that glycogen- and amylopectin-debranching enzymes, such as pullulanases and isoamylases, can be used as effective additives in dishwashing and laundry detergents (unpublished results), as also reported by us for alkaline endoglucanases from some strains of *Bacillus* (Okoshi *et al.*, 1990; Yoshimatsu *et al.*, 1990). We have isolated a strain of *Bacillus* that produces an alkaline pullulanase, the properties of which also fulfil the essential requirements for enzymes to be used for such purposes (Ara *et al.*, 1992). However, isoamylases of microbial origin reported to date cannot be used in alkaline detergents since they all have maximum activities at acidic pH values. This report describes the production, purification and properties of an alkaline isoamylase from an alkalophilic strain of *Bacillus* isolated from soil.

Methods

Organism and culture conditions. The organism used was *Bacillus* sp. KSM-3309, which was originally isolated from a soil sample collected in Mito, Japan. Its morphological and taxonomic characteristics were examined according to the methods of Gordon *et al.* (1973) and Claus & Berkeley (1986).

* Author for correspondence. Tel. 81 285 83 7400; fax 81 285 83 7403.

Bacillus sp. KSM-3309 was propagated at 30 °C in a medium composed of (w/v): 1.0% glycogen (from oyster; Sigma), 0.2% Tryptone (Difco), 0.1% yeast extract (Difco), 0.1% $(\text{NH}_4)_2\text{SO}_4$, 0.3% KH_2PO_4 , 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0001% $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ and 0.5% Na_2CO_3 (pH 9.2). Solutions of CaCl_2 and Na_2CO_3 were autoclaved separately. The organism was grown with shaking at 30 °C for 3 d in 100 ml aliquots of medium in 500 ml flasks. Cells were harvested by centrifugation (9800 g, 30 min) at 4 °C. All further manipulations were also done at this temperature. The supernatant obtained was used for purification of the enzyme.

Purification of the enzyme. The supernatant from a 3 litre culture was treated with ammonium sulphate, and the fraction that precipitated at 80% saturation was collected by centrifugation (12000 g, 20 min). The precipitate was dissolved in 10 mM-Tris/HCl buffer (pH 8.0) and the solution was dialysed twice over the course of 16 h against 50 vols of the same buffer. The retentate was diluted to 100 ml with 10 mM-Tris/HCl (pH 8.5) and mixed with DE52-cellulose powder (1 g; Whatman) that had been equilibrated in the same buffer. After the suspension had been mixed gently for 30 min, the support was collected on a membrane (PM-10, 100000- M_r cutoff; Amicon) and washed with 10 mM-Tris/HCl buffer. Proteins were then eluted with the same buffer supplemented with 1.0 M-NaCl. The eluate obtained was concentrated by ultrafiltration (PM-10) and directly applied to a column of DEAE-Bio-Gel A (1.5 cm $\times$ 60 cm; Bio-Rad) that had been equilibrated with 10 mM-Tris/HCl buffer (pH 8.0). The column was first washed with 300 ml of the equilibrating buffer and proteins were eluted with a 1.0 litre linear gradient of 0 to 1.0 M-NaCl in the same buffer, at a flow rate of 40 ml h^{-1} . Fractions of 1.5 ml were collected from the start of the gradient. The active fractions (tubes 272–283) were combined and concentrated by ultrafiltration on a PM-10 membrane. The concentrate (1 ml) was loaded directly on a column of Sephacryl S-200 (1.5 cm $\times$ 96 cm; Pharmacia) that had been equilibrated with 10 mM-Tris/HCl buffer (pH 8.0) containing 0.1 M-NaCl. Elution was done with the same buffer; 1.5 ml fractions were collected at a flow rate of 7 ml h^{-1} . The elution pattern was monitored by measuring protein (absorbance at 280 nm) and isoamylase activity in each fraction. Only one peak of isoamylase activity (fractions 57–62) was eluted. The active fractions were combined and dialysed overnight against a large volume of 10 mM-Tris/HCl buffer (pH 8.0), and the retentate was used for further experiments as the final preparation of purified enzyme.

Enzyme assay. The routine assay for isoamylase activity using oyster glycogen as substrate was performed with 50 mg substrate and a sample of enzyme in 1.0 ml 40 mM-glycine/NaCl/NaOH buffer (pH 9.0); reactions were incubated at 40 °C for 15 min. The reducing sugar formed was quantified as glucose by the dinitrosalicylic acid procedure (Miller *et al.*, 1960). One unit (U) of enzyme activity was defined as the amount of protein that produced 1 μmol product min^{-1} under these conditions. Isoamylase activity was also followed with amylopectin as substrate, by determining the increase in iodine staining power (Maruo & Kobayashi, 1951). The reaction mixture consisted of 0.5 ml 2.0% (w/v) potato amylopectin (Sigma) in 0.2 M-glycine/NaCl/NaOH buffer (pH 9.0) and 0.5 ml enzyme solution. At intervals, 0.2 ml aliquots of the reaction mixture were mixed with 2.0 ml aliquots of iodine solution (0.005% I_2 plus 0.015% KI), and each mixture was diluted to 8.0 ml with deionized water. One unit of isoamylase activity was defined as the amount of protein that caused an increase in absorbance at 600 nm of 0.01 min^{-1} under the standard assay conditions. Protein was quantified by the procedure of Bradford (1976) using the Bio-Rad protein assay kit, with bovine serum albumin as the standard.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulphate (SDS)-PAGE were done using 7.5% (w/v) and 15% acrylamide slab gels, respectively, as described elsewhere (Ara *et al.*, 1992). Isoelectric focusing of the purified enzyme was done by

PAGE, as described by Wrigley (1971). The enzyme (10 μg) was subjected to electrophoresis on a column (0.5 cm $\times$ 8.5 cm) of 7.5% acrylamide gel that contained 13.3% (v/v) glycerol and 1.9% (v/v) Pharmalyte (pH range 3–10; Pharmacia), at a constant voltage of 300 V for 14 h at 4 °C, with the cathode in the upper chamber (20 mM- H_3PO_4) and the anode in the lower one (0.1 M-NaOH). The isoelectric point was determined by cutting out zones of gel, soaking them in deionized water and measuring the pH of the resultant solution.

Determination of molecular mass. Molecular mass was estimated both by gel filtration on a column of Sephacryl S-200 and by SDS-PAGE. The Sephacryl column (1.5 cm $\times$ 96 cm) was equilibrated with 10 mM-Tris/HCl buffer (pH 8.0) supplemented with 0.1 M-NaCl and calibrated by elution of molecular-mass markers (Pharmacia), which included hen egg white ovalbumin (42.7 kDa), bovine serum albumin (66.2 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), rabbit muscle aldolase (158 kDa), bovine liver catalase (232 kDa) and horse spleen ferritin (440 kDa). For the determination of molecular mass by SDS-PAGE, molecular mass markers for SDS-PAGE (Bio-Rad) were used: soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31 kDa), hen egg white ovalbumin (42.7 kDa), bovine serum albumin (66.2 kDa) and rabbit muscle phosphorylase *b* (97.4 kDa).

Chromatographic analysis of the products of hydrolysis of polysaccharides. Excess purified enzyme (15 mU) was incubated at 40 °C for an appropriate length of time (up to 12 h) in 2.0 ml of 40 mM-Tris/HCl buffer (pH 8.0) with 0.5 mg of the following polysaccharides: pullulan (Hayashibara), potato amylose (DP = 17; Wako Pure Chemical), corn amylopectin (Sigma), potato amylopectin, oyster glycogen, bovine muscle glycogen (Sigma), or rabbit liver glycogen (Sigma). At intervals, 10 μl aliquots were taken and used for ascending paper chromatography (on no. 50 filter paper; Toyo Roshi) in a solvent system of 1-butanol/pyridine/water (6:4:3, by vol.). The chromatograms were developed by spraying with silver nitrate reagent (Trevelyan *et al.*, 1950).

Results

Taxonomic characterization of strain KSM-3309

The isolate grew well at an initial pH between 8.5 and 10.5 (pH adjusted with Na_2CO_3) but could not grow below pH 8.0 in nutrient broth containing 1.0% (w/v) glucose, i.e. it was an obligate alkalophile. Therefore, media used for identification were supplemented with 0.5% (w/v) Na_2CO_3 . The organism was an obligate aerobe, which was spore-forming (cylindrical, central endospores), Gram-positive, motile, and rod-shaped (0.8–1.0 μm $\times$ 2.0–2.5 μm) with peritrichous flagella. It was positive for reduction of nitrate, production of catalase and hydrolysis of starch, and negative for formation of acetoin (V-P test), production of urease and oxidase, and formation of H_2S and indole. It seems likely that strain KSM-3309 is a relative of *Bacillus circulans*.

Purification of the enzyme

When *Bacillus* sp. KSM-3309 was grown in medium containing 1.0% (w/v) oyster glycogen, isoamylase activity, determined by the iodine staining procedure,

Fig. 1. Gel chromatography of isoamylase on the final column of Sephacryl S-200. Isoamylase activity in each 1.5 ml fraction was measured by both the dinitrosalicylic acid (DNS) (●) and iodine staining (○) methods, and the protein concentration was monitored as A_{280} (....).

was maximal after 3 d (304 U ml^{-1}). After the final chromatography of the crude enzyme on Sephacryl S-200, a single peak of isoamylase activity was detected when fractions were assayed by both the dinitrosalicylic acid and iodine coloration methods (Fig. 1). The degree of purification and total recovery are summarized in Table 1. Approximately 166-fold purification was achieved, with an overall yield of 6.8% and a specific activity of $469 \text{ U (mg protein)}^{-1}$. When the iodine coloration method was used, the purified enzyme had a specific activity of $39.4 \text{ U (mg protein)}^{-1}$. The characterization of the isoamylase was performed exclusively with the final preparation.

Homogeneity, absorption spectrum, molecular mass and isoelectric point

The purified enzyme preparation was tested for purity by nondenaturing PAGE and SDS-PAGE, which both gave

Fig. 2. Nondenaturing PAGE (a) and SDS-PAGE (b) of the purified isoamylase from *Bacillus* sp. KSM-3309. PAGE was done on a 7.5% (w/v) polyacrylamide slab gel (1 mm thickness) with 5 mM-Tris/38 mM-glycine/NaOH buffer (pH 8.5) as the running buffer. SDS-PAGE was done on a 15% (w/v) polyacrylamide slab gel with 25 mM-Tris/glycine buffer (pH 8.3) containing 0.1% (w/v) SDS as the running buffer, after the enzyme had been heated at 95°C for 5 min in 62.5 mM-Tris/HCl buffer (pH 6.8) containing 20% (w/v) SDS, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol. Approximately $7 \mu\text{g}$ of the purified enzyme was used for electrophoresis in each case. The bands of protein were visualized by silver staining (Oakley *et al.*, 1980).

a single band of protein when proteins were visualized by silver staining (Fig. 2). The purified enzyme had an absorption maximum at 280 nm due to the presence of a simple protein, with an absorption coefficient ($A_{1\text{cm}}^{1\%}$ at 280 nm) of 11.8. The molecular mass of the enzyme was estimated to be 65 kDa by gel filtration using Sephacryl S-200. SDS-PAGE gave the same molecular mass of 65 kDa for subunits of the enzyme (Fig. 2). These results indicate that the purified enzyme has a monomeric structure. The isoelectric point of the enzyme was estimated to be pH 4.2 by isoelectric focusing (see Methods).

Table 1. Purification of the isoamylase from *Bacillus* sp. KSM-3309

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (-fold)
Cell-free culture	390	1100	2.82	100	1.0
Ammonium sulphate	25.3	811	32.1	74	11.4
DE52-cellulose	13.6	597	43.9	54	15.6
DEAE-Bio-Gel A	1.22	205	168	19	59.6
Sephacryl S-200	0.16	75	469	6.8	166

Table 2. *Substrate specificity of the isoamylase*

Assays were performed at 40 °C in 40 mM-glycine/NaCl/NaOH buffer (pH 9.0) with 0.17 g of enzyme under the standard reaction conditions.

Substrate (0.25 %, w/v)	Isoamylase activity (U mg ⁻¹)	Relative activity (%)
Glycogen (oyster)	470	100
Glycogen (bovine muscle)	522	111
Glycogen (rabbit liver)	369	78.6
Amylopectin (potato)	555	118
Amylopectin (corn)	667	142
Pullulan	7.4	1.57
Amylose (potato, DP = 17)	0	0
Maltose*	0	0

* Glucose generated from maltose (10 mM) was quantified by the glucose/peroxidase procedure (Glucose C-Test; Wako Pure Chemical).

Fig. 3. Changes in the absorption spectrum of the iodine-staining solution when glycogen (a) and amylopectin (b) were treated with the alkaline isoamylase. Oyster glycogen and potato amylopectin (50 mg each) were incubated at 40 °C with purified enzyme (43 mU) in a final volume of 5.0 ml in 40 mM-Tris/HCl buffer (pH 8.0). At the times indicated on the spectra, 1.0 ml aliquots were withdrawn and mixed with 4 ml iodine solution (0.002 % I₂/0.02 % KI). The samples were diluted to 8.0 ml with deionized water and used for determination of the absorption spectra of the iodine complex.

Substrate specificity

The purified enzyme preparation was examined for its ability to hydrolyse various carbohydrates at 40 °C and at pH 9.0 in 40 mM-glycine/NaCl/NaOH buffer (Table 2). Of the carbohydrate substrates tested, glycogens (from oyster, rabbit liver and bovine muscle) and amylopectins (from potato and corn) were efficiently hydrolysed at similar rates. In the products of hydrolysis of these substrates, glucose and maltodextrins (maltose to maltohexaose) were not detectable after paper chromatography of the hydrolysates (for details of the

chromatographic procedure, see Methods). The enzyme had an apparent K_m value of 0.59 mg ml⁻¹ for potato amylopectin, with a V_{max} of 588 U (mg protein)⁻¹; and a K_m of 0.45 mg ml⁻¹ for oyster glycogen, with a V_{max} of 645 U (mg protein)⁻¹. Pullulan (α -1,6-linkage), amylose (α -1,4-linkage), and maltose were practically unaffected by the enzyme.

Fig. 3 shows the changes in absorption spectra of the iodine-staining solution for products of potato amylopectin and oyster glycogen generated during their treatment with the enzyme. With both substrates, the absorbance increased and the maximum wavelength of absorption shifted to a longer wavelength (from 530 to 550 nm) as the enzyme reaction proceeded. These results suggest that this enzyme can hydrolyse the 1,6- α -glucosidic linkages of branched α -D-glucans and that it differs from pullulanases (EC 3.2.1.41), which can hydrolyse pullulan to generate oligosaccharides (Bender & Wallenfels, 1961; Walker, 1968; Nakamura *et al.*, 1987; Ara *et al.*, 1992). It seems appropriate to refer to the enzyme described in this report as an isoamylase.

Effects of pH on the activity and stability of the enzyme

The effect of pH on the activity of the purified isoamylase was determined at 40 °C in various buffers between pH 4.0 and pH 12.0 (Fig. 4). Maximum activity toward oyster glycogen was observed at around pH 9.0 in 40 mM-glycine/NaCl/NaOH buffer. The enzyme was active between pH 5.0 and pH 11.5, with more than 65 % of the maximum activity being detectable at pH 10. To determine the stability of the enzyme activity to changes in pH, the isoamylase was preincubated overnight at 4 °C in various buffers and then assayed at 40 °C and pH 9.0 in 0.1 M-glycine/NaCl/NaOH buffer. The enzyme activity remained unchanged during incubations in buffers at pH 7.5 to pH 9.5 (Fig. 4). More than 50 % of the original activity was detectable after incubation in buffers at pH values between 6.6 and 10.5.

Effects of temperature on the activity and stability of the enzyme

The activity of the alkaline isoamylase with glycogen as substrate was determined at various temperatures in 40 mM-glycine/NaCl/NaOH buffer (pH 9.0). Maximum activity with oyster glycogen as substrate was detected at around 55 °C. At temperatures below 30 °C and above 65 °C the enzyme was almost inactive. The activity of the enzyme was also examined after it had been heated at various temperatures for 30 min in 40 mM-glycine/NaCl/NaOH buffer (pH 9.0). The enzyme was stable up to 40 °C. Above this temperature, the stability of the enzyme gradually decreased, with less than 5 % of the

Fig. 4. Effects of pH on the activity and stability of the purified isoamylase. The buffers used were: acetate, pH 4.0–5.5; Tris/maleate, pH 5.5–8.5; glycine/NaCl/NaOH, pH 8.5–10.5; borate, pH 10.5–12.0. The isoamylase activity (●) was measured at 40 °C in the indicated buffers at 40 mM, at various pH values, with 0.17 µg enzyme and oyster glycogen as substrate. Enzyme activity at pH 9.0 in 40 mM-glycine/NaCl/NaOH buffer was taken as 100%. The stability of the enzyme (○) was examined in buffers of various pH. The enzyme (1.7 µg) was incubated at the indicated pH in 1.0 ml 10 mM-buffer overnight at 4 °C, then aliquots of 0.1 ml were withdrawn and the residual isoamylase activity was measured at 40 °C and pH 9.0 in 0.1 M-glycine/NaCl/NaOH buffer. The values shown are the percentages of the original activity.

original activity remaining after 30 min incubation at 60 °C (data not shown).

Effects of chemical reagents

The activity of alkaline isoamylase, with oyster glycogen as substrate, was examined after the enzyme had been incubated with various chemical reagents (each 1 mM) in 10 mM-glycine/NaCl/NaOH buffer (pH 8.0) at 40 °C for 15 min. An aliquot (0.1 ml) was then used to assay the residual isoamylase activity in 0.1 M-glycine buffer. The enzyme was not significantly affected by the following cations: Na⁺, K⁺, Mg²⁺, Ca²⁺, Mn²⁺, Ni²⁺, Co²⁺, Cu²⁺, Cd²⁺, Ba²⁺, Fe²⁺, Fe³⁺ and Al³⁺. The notable exception was Hg²⁺ (52% inhibition). However, the thiol inhibitors *N*-ethylmaleimide (at 20 °C), monoiodoacetate (at pH 5.0 in 10 mM-acetate buffer) and 4-chloromercuribenzoate (at pH 5.0 in 10 mM-acetate buffer), either had no effect or caused very low levels of inhibition. *N*-Bromosuccinimide (10 µM) abolished enzyme activity almost completely, after incubation of the enzyme at 4 °C and pH 5.0 in 10 mM-acetate buffer with this compound for 15 min. EDTA (10 mM), phenylmethane-sulphonyl fluoride (1 mM), α-cyclodextrin (10 mM) and β-cyclodextrin (10 mM) were not inhibitory under the standard assay conditions.

Discussion

Members of the genus *Bacillus* produce various kinds of extracellular enzyme of industrial importance, including amylases, proteinases, endoglucanases and pectinases.

In the present study, we isolated an obligately aerobic and alkalophilic strain of *Bacillus*, KSM-3309, which produces an isoamylase with a pH optimum of 9.0. Under the alkaline reaction conditions used in this study, this enzyme efficiently hydrolysed amylopectin and glycogen, but it had no appreciable activity against pullulan or amylose. The colour generated by iodine-staining of digests of amylopectin and glycogen changed to blue, with a shift in the absorption maximum from 530 nm to 550 nm, and the absorption also increased significantly (see Fig. 3). These results suggest that the action of the alkaline enzyme on amylopectin and glycogen resulted in specific cleavage of α-1,6-glucosidic inter-chain linkages, producing essentially linear chains.

The isoamylase from *Bacillus* sp. KSM-3309 shows activity and stability at the highest pH reported thus far for isoamylases from micro-organisms and higher plants. Acid and neutral isoamylases have been reported from *Pseudomonas amyloclavata* (Yokobayashi *et al.*, 1970), *Cytophaga* sp. (Gunja-Smith *et al.*, 1970a), *Bacillus amyloliquefaciens* (Urlaub & Wober, 1975), *E. coli* (Jeanningros *et al.*, 1975), *Flavobacterium* sp. (Sato & Park, 1980), the yeast *Lipomyces kononenkoae* (Spencer-Martins, 1982) and potato tubers (*Solanum tuberosum* L.; Ishizuka *et al.*, 1983), the maximum pH for activity of these enzymes being 3.5, 5.5, 5.0, 5.6–6.4, 6.3, 5.6 and 5.5–6.0, respectively.

The alkaline isoamylase from *Bacillus* sp. KSM-3309 was inhibited by Hg²⁺ ions but not by the thiol inhibitors iodoacetate, 4-chloromercuribenzoate and *N*-ethylmaleimide. Thus, the inhibition by Hg²⁺ ions is unlikely to be due to interactions with cysteine residues, but it could involve interactions with tryptophan residue(s). The isoamylase activity was completely abolished by a low concentration (10 µM) of *N*-bromosuccinimide, which oxidizes tryptophan residues (Eriksson & Pettersson, 1968). It has been suggested that a histidine residue may be important for the activity of an isoamylase from *Pseudomonas* (Kitagawa *et al.*, 1975). Our isoamylase was not inhibited by β-cyclodextrin, as was also true for an isoamylase from *L. kononenkoae* (Spencer-Martins, 1982). Marshall (1973) showed that cyclodextrins do not inhibit the isoamylase activity of *Cytophaga* but strongly inhibit the pullulanase activities of *Aerobacter* and sweet potato. He suggested that an examination of sensitivity toward cyclodextrins might provide a simple method for differentiating isoamylases from pullulanases. Our data tend to support his suggestion.

Amemura *et al.* (1988) and Tognoni *et al.* (1989) cloned and sequenced the genes for the isoamylases from *P. amyloclavata* SB-15, which had been isolated by Harada *et al.* (1968), and *Pseudomonas* sp. SMP1, respectively. They found that the deduced amino acid sequences of isoamylases from strains SB-15 and SMP1 both showed considerable homology of those of α -amylases, pullulanases and a cyclodextrin glucanotransferase (EC 2.4.1.19). Homology was especially conspicuous in the regions that included the proposed catalytic and substrate-binding sites. The sequences of the two pseudomonad enzymes also have several regions that are similar to regions in a neopullulanase isolated from the alkalophilic *Bacillus* sp. KSM-1876 (Igarashi *et al.*, 1992). It is of interest that the isoamylases of strains SB-15 and SMP1 have optimum pH values for activity between 3 and 4, while the optimum pH is 9.0 for the enzyme from *Bacillus* sp. KSM-3309. The deduced amino acid sequence of this alkaline enzyme may be helpful in identifying protein motifs that determine the pH optima for isoamylases of microbial origin. Current work is focussing on the cloning and sequencing of the alkaline isoamylase gene from *Bacillus* sp. KSM-3309.

References

- AKAI, H., YOKOBAYASHI, K., MISAKI, A. & HARADA, Y. (1971). Complete hydrolysis of branching linkages in glycogen by pseudomonas isoamylase: distribution of linear chains. *Biochimica et Biophysica Acta* **237**, 422–429.
- AMEMURA, A., CHAKRABORTY, R., FUJITA, M., NOUMI, T. & FUTAI, M. (1988). Cloning and nucleotide sequence of the isomylase gene from *Pseudomonas amyloclavata* SB-15. *Journal of Biological Chemistry* **263**, 9271–9275.
- ARA, K., IGARASHI, K., SAEKI, K., KAWAI, S. & ITO, S. (1992). Purification and some properties of an alkaline pullulanase from alkalophilic *Bacillus* sp. KSM-1876. *Bioscience, Biotechnology, and Biochemistry* **56**, 62–65.
- BENDER, H. & WALLENFELS, K. (1961). Untersuchungen an Pullulan. II. Spezifischer Abbau durch ein bakterielles Enzym. *Biochemische Zeitschrift* **334**, 79–95.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- CLAUS, D. & BERKELEY, R. C. W. (1986). Genus *Bacillus*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- ERIKSSON, K.-E. & PETERSSON, G. (1968). Studies on cellulolytic enzymes. V. Some structural properties of the cellulase from *Penicillium notatum*. *Archives of Biochemistry and Biophysics* **124**, 160–166.
- GORDON, R. E., HAYNES, W. C. & PANG, C. H. (1973). The Genus *Bacillus*. Washington, DC: US Department of Agriculture.
- GUNJA, Z. H., MANNERS, D. J. & MAUNG, K. (1961). Studies on carbohydrate-metabolizing enzymes. VII. Yeast isoamylase. *Biochemical Journal* **81**, 392–398.
- GUNJA-SMITH, Z., MARSHALL, J. J., MERCIER, C., SMITH, E. E. & WHELAN, W. J. (1970a). A glycogen-debranching enzyme from *Cytophaga*. *FEBS Letters* **12**, 96–100.
- GUNJA-SMITH, Z., MARSHALL, J. J., MERCIER, C., SMITH, E. E. & WHELAN, W. J. (1970b). A reversion of the Mayer–Bernfeld model of glycogen and amylopectin. *FEBS Letters* **12**, 101–104.
- HARADA, T., YOKOBAYASHI, K. & MISAKI, A. (1968). Formation of isoamylase by *Pseudomonas*. *Applied Microbiology* **16**, 1439–1444.
- HARADA, T., MISAKI, A., AKAI, K., YOKOBAYASHI, K. & SUGIMOTO, K. (1972). Characterization of *Pseudomonas* isoamylase by its actions on amylopectin and glycogen: comparison with *Aerobacter pullulanase*. *Biochimica et Biophysica Acta* **268**, 497–505.
- HIZUKURI, S., KANENO, T. & TAKEDA, I. (1983). Measurement of the chain length of amylopectin and its relevance to the origin of crystalline polymorphism of starch granules. *Biochimica et Biophysica Acta* **760**, 188–191.
- IGARASHI, K., ARA, K., SAEKI, K., OZAKI, K., KAWAI, S. & ITO, S. (1992). Nucleotide sequence of the gene that encodes a neopullulanase from an alkalophilic *Bacillus*. *Bioscience, Biotechnology, and Biochemistry* **56**, 514–516.
- ISHIZUKA, Y., TANIGUCHI, H., MARUYAMA, Y. & NAKAMURA, M. (1983). Debranching enzymes of potato tubers (*Solanum tuberosum* L.). I. Purification and some properties of potato isoamylase. *Agricultural and Biological Chemistry* **47**, 771–779.
- JEANINGROS, R., CREUZET, N., FRISON, C. & CATTANEO, J. (1975). A debranching enzyme in *Escherichia coli*. *Biochemical Society Transactions* **3**, 336–337.
- KITAGAWA, H., AMEMURA, A. & HARADA, T. (1975). Studies on the inhibition and molecular properties of crystalline *Pseudomonas* isoamylase. *Agricultural and Biological Chemistry* **39**, 989–994.
- MARSHALL, J. J. (1973). Inhibition of pullulanase by Schardinger dextrans. *FEBS Letters* **37**, 269–273.
- MARUO, B. & KOBAYASHI, T. (1951). Enzymic scission of the branch links in amylopectin. *Nature, London* **167**, 606–607.
- MILLER, G. L., BLUM, R., GLENNON, W. E. & BURTON, A. L. (1960). Measurement of carboxymethylcellulase activity. *Analytical Biochemistry* **2**, 127–132.
- NAKAMURA, N., SASHIHARA, N., NAGAYAMA, H. & HORIKOSHI, K. (1987). Production of a thermostable pullulanase by *Thermus* sp. *Journal of the Japanese Society of Starch Science* **34**, 38–44.
- NORMAN, B. E. (1982). A novel debranching enzyme for application in the glucose syrup industry. *Starch/Stärke* **34**, 340–346.
- OAKLEY, B. R., KIRSCH, D. R. & MORRIS, N. R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analytical Biochemistry* **105**, 361–363.
- OKOSHI, H., OZAKI, K., SHIKATA, S., OSHINO, K., KAWAI, S. & ITO, S. (1990). Purification and characterization of multiple carboxymethylcellulases from *Bacillus* sp. KSM-522. *Agricultural and Biological Chemistry* **54**, 83–89.
- SATO, H. H. & PARK, Y. K. (1980). Purification and characterization of extracellular isoamylase from *Flavobacterium* sp. *Starch/Stärke* **32**, 132–136.
- SPENCER-MARTINS, I. (1982). Extracellular isoamylase produced by the yeast *Lipomyces kononenkoae*. *Applied and Environmental Microbiology* **44**, 1253–1257.
- STOMINSKA, L. & MACZYNSKI, M. (1985). Studies on the application of pullulanase in starch saccharification process. *Starch/Stärke* **37**, 386–390.
- TOGNONI, A., CARRERA, P., GALLI, G., LUCCHESI, G., CAMERINI, B. & GRANDI, G. (1989). Cloning and nucleotide sequence of the isoamylase gene from a strain of *Pseudomonas* sp. *Journal of General Microbiology* **135**, 37–45.
- TREVELYAN, W. E., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, London* **166**, 444–445.
- URLAUB, H. & WOBER, G. (1975). Identification of isoamylase, a glycogen-debranching enzyme, from *Bacillus amyloliquefaciens*. *FEBS Letters* **57**, 1–4.
- WALKER, G. J. (1968). Metabolism of the reserve polysaccharide of *Streptococcus mitis*. *Biochemical Journal* **108**, 33–40.
- WRIGLEY, C. W. (1971). Gel electrofocusing. *Methods in Enzymology* **22**, 559–564.
- YOKOBAYASHI, K., MISAKI, A. & HARADA, T. (1970). Purification and properties of pseudomonas isoamylase. *Biochimica et Biophysica Acta* **212**, 458–469.
- YOSHIMATSU, T., OZAKI, K., SHIKATA, S., OHTA, Y., KOIKE, K., KAWAI, S. & ITO, S. (1990). Purification and characterization of alkaline endo-1,4- β -glucanases from alkalophilic *Bacillus* sp. KSM-635. *Journal of General Microbiology* **136**, 1973–1979.