Carbohydrate Research, 197 (1990) 227–235
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION AND PROPERTIES OF AN OLIGO-1,6-D-GLUCOSIDASE FROM AN ALKALOPHILIC *Bacillus* SPECIES

MIKIO YAMAMOTO* AND KOKI HORIKOSHI
The RIKEN Institute, 2-1, Hirosawa, Wako, Saitama 351-01 (Japan)
(Received January 11th, 1989; accepted for publication, May 22nd, 1989)

ABSTRACT

An isomaltose-hydrolyzing α-D-glucosidase from the alkalophilic *Bacillus* designated strain F5 was purified to an electrophoretically homogeneous state. The molecular weight of the purified glucosidase was 60 000 by SDS–poly(acrylamide) gel electrophoresis, and 63 000 by Sephacryl S-200 gel-filtration chromatography. The enzyme was most active for isomaltose at pH 6.0–6.5 and 45°, and stable up to 50° at pH 7.0 and in the range of pH 6.0–9.0 at 50° by 10-min incubation. The apparent V_{max} and K_m values for isomaltose were 34.5 μmol.min^{-1}.mg^{-1} of protein, and 3.33mM. Panose and isomaltotriose are the best substrates for this enzyme. The restricted substrate specificity indicated the assignment of the enzyme to be an oligo-1,6-glucosidase (dextrin 6-α-glucanohydrolase; EC 3.2.1.10), but it was suggested that it could be a new type of oligo-1,6-glucosidase on the basis of its action on a series of (1→4)-α-malto-oligosaccharides.

INTRODUCTION

Three types of enzyme hydrolyzing 1,6-α-D-glucosidic linkages are known, *i.e.*, (*a*) oligo-1,6-D-glucosidase[1–6] [EC 3.2.1.10] hydrolyzes (1→6)-α-D-glucosidic linkages in isomaltose and dextrins produced from starch and glycogen by alpha amylase, (*b*) amylo-(1→6)-glucosidase[7,8] [EC 3.2.1.33] endohydrolyzes (1→6)-α-D-glucosidic linkages at points of branching in chains of (1→4)-linked α-D-glucosyl residues, and (*c*) exo-1,6-α-D-glucosidase[9,10] [EC 3.2.1.70] successively hydrolyzes D-glucosyl residues from 1,6-α-D-glucans and derived oligosaccharides. Some kinds of α-D-glucosidase[11–13] [EC 3.2.1.20] also show only a little activity to (1→6)-α-D-glucosidic linkages.

Previously[14], we reported on the characterization of an alkalophilic soil bacterium which produces intracellular, isomaltose-hydrolyzing α-D-glucosidase in an alkalophilic medium containing 1% of $NaHCO_3$. This crude enzyme showed a strict substrate specificity to the (1→6)-α-D-glucosidic linkage of disaccharides com-

*Present address: Research Laboratory of Nihon Shokuhin Kako Co., 30 Tajima, Fuji, Shizuoka 417, Japan.

0008-6215/90/$ 03.50

posed of D-glucose units. We now deal with the purification procedure and some properties of the enzyme of this strain (alkalophilic *Bacillus* strain F5).

MATERIALS AND METHODS

Micro-organism. — An alkalophilic *Bacillus* strain F5 (FERM P-7497) was maintained on an isolation-medium agar slant[14] and stored at room temperature.

Materials. — Isomaltose, panose, isomaltitol, maltitol, and maltotriitol were obtained from Hayashibara Biochemical Lab., Japan. *p*-Nitrophenyl α-D-glucopyranoside (pNPG) was from Sigma. Isopanose was kindly donated by Dr. Y. Sakano of Tokyo University of Agriculture and Technology. D-Glucosyl- and maltosyl-α-cyclodextrin (CD) were obtained from Prof. S. Hizukuri of Kagoshima University. Series of malto-oligosaccharides and pNP-malto-oligosaccharides were products of Nihon Shokuhin Kako Co., Japan. A series of isomalto-oligosaccharides was prepared by high performance liquid chromatography (h.p.l.c.) from a dextranase digest of dextran 70 (Meito Sangyo Co., Japan). Isomaltotriitol and panitol were prepared by reduction of isomaltotriose and panose with $NaBH_4$.

Commercially available soluble starch was washed twice with distilled water and acetone before use. Polypeptone and yeast extract were respectively obtained from Nihon Seiyaku Co., Japan, and Difco Lab., U.S.A.

Glucostat (Worthington) reagent, lysozyme, and deoxyribonuclease (DNase) were purchased from Fujisawa Pharm. Ind. Co. (Japan), Seikagaku Kogyo Co. (Japan), and Miles Lab. (U.S.A.), respectively.

All other chemicals used were of the highest quality available commercially.

Enzyme assay. — Isomaltose-hydrolyzing α-D-glucosidase activity was determined as follows: The reaction mixture, consisting of isomaltose (3.6 mg) in 0.45 mL of 50mM sodium phosphate (Na-P) buffer (pH 6.8) and 0.05 mL of the enzyme solution, was incubated for 10 min at 40°. After terminating the reaction by heating for 3 min in a boiling-water bath, the D-glucose formed was determined enzymically by the D-glucose oxidase–peroxidase method[15]. One unit (U) of the enzyme activity is defined as the amount of enzyme that hydrolyzes 1 μmol of isomaltose per min under the conditions just described.

Determination of protein concentration. — Protein was determined by the method of Lowry *et al.*[16], with bovine serum albumin (Sigma) as the standard.

Preparation of the crude enzyme. — The alkalophilic *Bacillus* sp. F5 was cultured aerobically, in 7 L of the isolation medium described, under the following conditions: temperature, 37°; aeration, 3.5 L per min; and agitation, 250 r.p.m. After cultivation for 24 h, the cells (184 g wet weight, 21.7 U/g wet cells) were harvested by centrifugation at 6000*g* for 20 min at 4°, and then washed twice with 20mM Na-P buffer (pH 6.8) containing 0.9% of NaCl. The washed cells were suspended in 350 mL of the same buffer containing 0.9% of NaCl, and the suspension was incubated for 30 min at 40° with lysozyme (1.75×10^6 IU) and DNase (1.75×10^3 IU). The cell debris was removed by centrifugation at 25 000*g*

for 30 min at 4°, the clear, supernatant liquor was dialyzed overnight against tap water, and the crude glucosidase solution was mixed with 4 volumes of acetone at −20°. The precipitate formed was obtained by filtration and dried at room temperature *in vacuo*. This preparation did not show any other related amylolytic enzyme activities (such as alpha amylase[17], pullulanase[18], and isoamylase[19]). The enzyme also could not hydrolyze D-glucosyl- and maltosyl-α-CD.

Purification of the enzyme. — Unless otherwise noted, all purification steps were done at 4°.

Step 1. The crude enzyme solution in 20 mL of 50mM Na-P buffer (pH 6.8) was applied to a column (2.6 cm i.d. × 65 cm) of DEAE-Toyopearl 650M ion-exchange resin equilibrated with 50mM Na-P buffer (pH 6.8), and the column was thoroughly washed with the same buffer. The enzyme adsorbed on the resin was eluted with a linear gradient of 0–200mM NaCl in the same buffer at a flow rate of 1.0 mL/min. The D-glucosidase was eluted at ~130mM NaCl. The fractions containing D-glucosidase were pooled, and concentrated by ultrafiltration with AMICON PM-30.

Step 2. The concentrated enzyme (5.0 mL) was loaded onto a column (3.2 cm i.d. × 85 cm) of Toyopearl HW-55S equilibrated with 50mM Na-P buffer (pH 7.0) containing 200mM NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions (25 mL) showing D-glucosidase activity were collected, and concentrated by the same method. The concentrate was dialyzed overnight against 50mM Na-P buffer (pH 7.0).

Step 3. The dialyzate (20 mL) was loaded onto a column (1.6 cm i.d. × 45 cm) of DEAE-Toyopearl 650M resin equilibrated with 50mM Na-P buffer (pH 7.0), the column washed with the same buffer, and the enzyme eluted with a linear gradient of 0–200mM NaCl at a flow rate of 0.6 mL/min, and active fractions (48 mL) were collected, and concentrated by the usual method.

Step 4. The concentrate (3 mL) was passed through a column (2.2 cm i.d. × 87 cm) of Sephacryl S-200 superfine resin equilibrated with 50mM Na-P buffer (pH 7.0) containing 200mM NaCl at a flow rate of 0.3 mL/min. Active fractions (18 mL were collected, and concentrated by the same method. This enzyme was used for the following experiments.

Polyacrylamide gel electrophoresis. — Polyacrylamide gel electrophoresis (PAGE) of the enzyme was carried out by using a pH 8.9 poly(acrylamide) gel and a Tris-HCl (pH 8.3) buffer system[20]. Purified enzyme (~20 μg) was applied to each gel and run at a constant current of 2 mA per tube for 120 min. The gel was then stained for proteins with 0.5% Coomassie Brilliant Blue R250 in 7% acetic acid solution, and destained with 7% acetic acid and 10% methanol.

The molecular weight of the enzyme was estimated by the SDS–PAGE method[21]. Molecular weight protein standard kit, phosphorylase *b* (mol. wt. 92 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400) were obtained from BIO-RAD Labs.

Isoelectric focusing. — The isoelectric point (pI) of the enzyme protein was determined with Servalyt Precotes (Serva, pH range 4–7). The purified enzyme solution (5 μL), containing 5 μg of protein, was applied on the sample applicator. The gel was prefocused at 5.5 W for 30 min, and then the applicator was removed. Focusing was achieved at 5.5 W for 90 min, and the proteins were stained with Coomasie Blue.

The isoelectric focusing calibration kit for pI determination, containing pepsinogen (pI; 2.80), amyloglucosidase (3.50), D-glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), and human carbonic anhydrase B (6.55), was obtained from Pharmacia Biotechnology International AB.

RESULTS AND DISCUSSION

Purification and homogeneity of the enzyme. — The result of the steps of purification of isomaltose-hydrolyzing α-D-glucosidase is summarized in Table I. The purified enzyme gave a single band of protein by PAGE, SDS–PAGE, and isoelectric focusing (pI, 4.2). In 10mM Na-P buffer (pH 7.0), the enzyme showed a typical absorption spectrum of protein. The ratio of absorption at 280 to that at 260 nm was ~2:1. This value indicated the absence of nucleic acid. The molecular weight was calculated to be 60 000 by SDS–PAGE, and 63 000 by Sephacryl S-200 chromatography.

Effect of pH and temperature on activity and stability. — As shown in Fig. 1A, the D-glucosidase was most active at pH 6.0–6.5. The stability of the enzyme

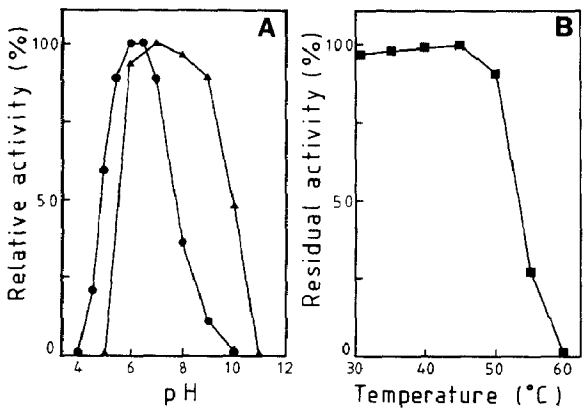

Fig. 1. Effect of pH (A) and temperature (B) on activity and stability. Symbols: optimum pH (●), pH stability (▲) and heat stability (■). The enzyme activities at various pH values were determined by the standard assay method. Residual activities were measured (A) after incubation at various pH values for 10 min at 50°, and (B) at various temperatures for 10 min at pH 7.0. Buffers used: 50mM citrate–sodium citrate (pH 4.0–6.0), 50mM Na_2HPO_4–NaH_2PO_4 (pH 6.0–8.0), 50mM glycine–NaCl–NaOH (pH 8.0–10.0), and 50mM Na_2HPO_4–NaOH (pH 11.0–12.0).

TABLE I

SUMMARY OF PURIFICATION OF ALKALOPHILIC *Bacillus* sp. F5 OLIGO-1,6-D-GLUCOSIDASE

Purification steps	*Total activity (U)*	*Yield (%)*	*Specific activity (U/mg of protein)*	*Purification*
All cell mass	4,590	—	—	—
Crude enzyme solution	2,920	64	—	—
Acetone ppt.	2,400	52	1.2	1
1st DEAE-Toyopearl	1,605	35	12.8	11
Toyopearl HW-55S	1,550	34	24.6	21
2nd DEAE-Toyopearl	1,120	24	27.9	23
Sephacryl S-200	972	21	34.2	29

at various pH values was also investigated (see Fig. 1A). The enzyme was stable in the pH range of 6.0–9.0 under these conditions.

The enzyme was most active at 45° (data not given). A solution of the enzyme in 50mM Na-P buffer (pH 7.0) was heated for 10 min at various temperatures, and the residual activity was measured (see Fig. 1B). The enzyme was stable up to 50°, but lost all activity at 60°.

The purified enzyme was most active at pH 6.0–6.5, and was stable from pH 6.0 to 9.0 after incubation for 10 min at 50°. As noted elsewhere[22–24], the inner pH of the cells of alkalophilic microorganisms is neutral. Accordingly, it is natural that the purified α-D-glucosidase is most active under neutral conditions.

Effects of metal ions and chemical reagents on activity. — Prolonged dialysis of the enzyme was carried out against 50mM HEPES buffer (pH 6.8). The residual activities after incubating for 30 min with various metal ions at 37°, or chemical reagents at 25°, and dialyzing overnight at 4° against the same buffer were measured by the standard assay method.

Results are expressed as percent residual activity compared with the activity in the control. As shown in Table II, the enzyme activity was strongly inhibited by such SH-group reagents as monoiodoacetate and *p*-chloromercuribenzoate (pCMB), and by some heavy-metal ions, such as Hg^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, and Fe^{2+}. The enzyme activity was slightly inhibited by Co^{2+} and NaF, but other metal ions tested, and ethylenedinitrilotetraacetate (EDTA), scarcely inhibited the activity.

Most *Bacillus* oligo-1,6-D-glucosidases reported so far do not have any cysteine residues, and they are not inhibited by pCMB (with the exception of the enzyme from *B. coagulans*). Notably, F5 oligo-1,6-D-glucosidase contains cysteine (data not shown). The inhibition of the enzyme activity by such heavy-metal ions as Hg^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, and pCMB suggested that a sulfhydryl group present in the enzyme protein may contribute to the active site of the enzyme. This is one of the points that distinguishes the enzyme of alkalophilic *Bacillus* F5 from those of neutrophilic strains.

TABLE II

EFFECTS OF METAL IONS AND CHEMICAL REAGENTS ON F5 OLIGO-1,6-D-GLUCOSIDASE ACTIVITY[a]

Compound added	*Concentration (mM)*	*Residual activity (%)*
$HgCl_2$	1	0
$PbCl_2$	1	0
$CuCl_2$	1	0
$ZnCl_2$	1	1
$FeCl_2$	1	10
$CoCl_2$	1	87
$MnCl_2$	1	95
$BaCl_2$	1	99
$MgCl_2$	1	100
pCMB	0.1	0
ICH_2CO_2H	1	23
NaF	10	76
EDTA	10	92

[a]For experimental conditions, see text.

Substrate specificity. — The enzyme released D-glucose from panose and isomalto-oligosaccharides (2–6 D-glucose units). Maltooligosaccharides, except maltotriose, pNPG and pNP-malto-oligosaccharides, except pNP-maltotriose, were also hydrolyzed slightly by the enzyme, but the liberation of D-glucose was confirmed when trisaccharide composed of D-glucose units and pNP-maltotriose were used as substrates. The enzyme did not release *p*-nitrophenol from pNP-malto-oligosaccharides larger than pNPG.

These findings indicate that the enzyme can hydrolyze the compounds by liberating a single D-glucose unit from the nonreducing end of saccharides linked by (1→4)-α- or (1→6)-α-D-glucosidic linkages. Similar findings were obtained with a series of sugar alcohols.

Neither liberation of D-glucose nor increase in reducing power was observed when the D-glucosidase was incubated with soluble starch, dextrin, dextran, or pullulan.

TABLE III

THE KINETIC PARAMETERS OF F5 OLIGO-1,6-D-GLUCOSIDASE FOR VARIOUS SUBSTRATES

Substrate	K_m *(mM)*	V_{max} *(μmol/min/mg of protein)*	V_{max}/K_m *ratio*
Maltose	13.3	4.2	0.32
Maltotriitol	4.0	25.2	6.3
Isomaltose	3.3	34.5	10.5
Maltotriose	1.9	29.9	15.7
Isomaltotriose	1.7	31.8	18.7
Panitol	1.0	29.0	29.0
Panose	1.0	33.8	33.8

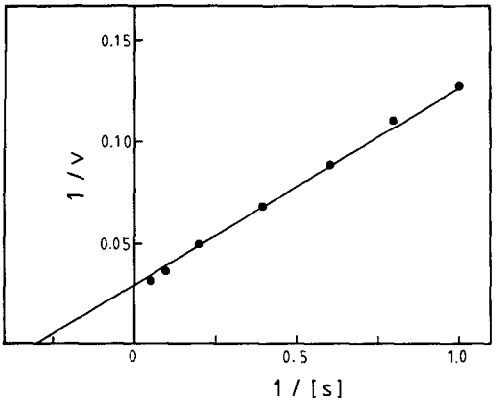

Fig. 2. Lineweaver–Burk plot for the determination of the Michaelis constant for isomaltose. The reaction rates were determined by the standard assay method at pH 6.8 and 40° with variation of the substrate concentration.

The purified enzyme demonstrated simple Michaelis–Menten kinetics with isomaltose, isomaltotriose, maltose, maltotriose, panose, maltotriitol, and panitol. The K_m and V_{max} ($= k_0$) values (see Table III) were calculated from Lineweaver–Burk plots. Fig. 2 shows the Lineweaver–Burk plot for isomaltose as an example. The reaction was done under the standard assay conditions by varying the substrate concentration. As shown in Table III, almost the same K_m values were observed with trisaccharides composed of D-glucose units, including panitol. Among them, panose and panitol were the best substrates for this enzyme.

Kinetic parameters also show the characteristic features of this enzyme. Almost the same values of K_m and k_0 were observed with trisaccharides composed of D-glucose units (without maltotriitol) whether the reducing end of them was free or had been reduced.

Some natural and synthetic saccharides that act as poor substrates for this enzyme act also as inhibitors whose inhibition types are somewhat varied.

The purified enzyme has action patterns similar to those of other oligo-1,6-D-glucosidase on several synthetic substrates and a series of isomalto-oligosaccharides, however, the hydrolysis rates of trisaccharides composed of D-glucose units, including panitol and maltotriitol, are very different from those of oligo-1,6-D-glucosidase of *S. mitis*[1], *L. bifidus*[2], hog intestinal mucosa[3], *B. thermoglucosidius*[4], *B. cereus*[5], and *B. coagulans*[6]. The data show that the alkalophilic *Bacillus* oligo-1,6-D-glucosidase has the highest affinity to trisaccharides composed of D-glucose units, with the exception of isopanose. This is also a point that may be used to characterize the enzyme of alkalophilic *Bacillus* F5.

Inhibition by sugar alcohols and malto-oligosaccharides. — Inhibition types and kinetic constants for some saccharides that were not hydrolyzed well by the D-glucosidase were investigated (see Table IV).

A series of pNP-malto-oligosaccharides and isomaltitol competitively

TABLE IV

INHIBITION OF THE ENZYME ACTIVITY BY SUGAR ALCOHOLS AND MALTO-OLIGOSACCHARIDES

Inhibitor	*Inhibition type*[a]	K_i (mM)
pNP-maltose	C	3.8
pNP-maltotriose	C	6.2
Isomaltitol	C	6.6
pNP-maltotetraose	C	15
pNP-maltopentaose	C	18
Maltitol	N	22
Maltose	N	32
Maltotetraose	N	35

[a]N, noncompetitive inhibition; C, competitive inhibition.

inhibited the enzyme ativity, and maltose, maltotetraose, and maltitol inhibited noncompetitively. Among them, pNP-maltose (K_i = 3.8mM), pNP-maltotriose (6.2mM), and isomaltitol (6.6mM) were relatively strong inhibitors toward the action of the D-glucosidase.

Kinetics of the simultaneous hydrolysis of panose and maltotriose. — An experiment in which the two substrates panose and maltotriose were simultaneously present was conducted in order to investigate whether this D-glucosidase hydrolyzes two substrates at the same catalytic site. Should this be the case, the following relationship would hold.

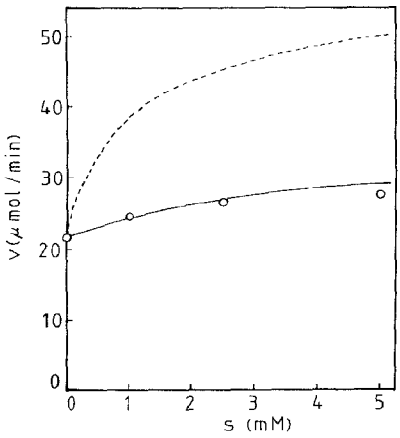

Fig. 3. Plot of *v vs. s* for mixed substrates. The concentration of maltotriose was fixed at 10mM, and the concentration of panose was varied from 0 to 5mM. Values of K_m and V_{max} used to calculate the theoretical values of he initial rate were, respectively, 1.9mM and 29.9 μmol/min/mg of protein for maltotriose, and 1.0mM and 33.8 μmol/min/mg of protein for panose. Symbols: ○, observed; solid line, calculated from the single-site model; dotted line, calculated from the two-site model.

$$v = \frac{V_A[A]}{K_A(1 + [B]/K_B) + [A]} + \frac{V_B[B]}{K_B(1 + [A]/K_A) + [B]} \quad (1)$$

in which A and B represent panose and maltotriose, [A] and [B] are the substrate concentrations of A and B, K_A and K_B are the Michaelis constant, K_m, for substrate A and B, V_A and V_B are the maximum velocity, V_{max}, for A and B, respectively, and v is the initial rate of the formation of D-glucose. Some typical plots of initial rate v against s (concentration of panose from 0 to 5mM) obtained for the mixed substrate solution at a fixed concentration of maltotriose (10mM) are shown in Fig. 3. The observed intial rates almost coincided with the v values calculated from Eq. *1*.

The result of this experiment strongly supports the view that this enzyme can hydrolyze glucosidic linkages of both linear and branched malto-trisaccharides at the same catalytic site in the special surroundings made by the shape and structure of the substrate and the enzyme.

REFERENCES

1 G. J. WALKER AND J. E. BUILDER, *Biochem. J.*, 105 (1967) 937–942.
2 R. W. BAILEY AND A. M. ROBERTON, *Biochem. J.*, 82 (1962) 272–277.
3 J. LARNER AND R. E. GILLESPIE, *J. Biol. Chem.*, 223 (1956) 709–726.
4 Y. SUZUKI, T. KISHIGAMI, AND S. ABE, *Appl. Environ. Microbiol.*, 31 (1976) 807–812.
5 Y. SUZUKI, R. AOKI, AND H. HAYASHI, *Biochim. Biophys. Acta*, 704 (1982) 476–483.
6 Y. SUZUKI AND Y. TOMURA, *Eur. J. Biochem.*, 158 (1986) 77–83.
7 T. E. NELSON, E. KOLB, AND J. LARNER, *Biochemistry*, 8 (1969) 1419–1428.
8 E. Y. C. LEE, J. H. CARTER, L. D. NIELSEN, AND E. H. FISCHER, *Biochemistry*, 9 (1970) 2347–2355.
9 G. J. WALKER AND A. PULKOWNIK, *Carbohydr. Res.*, 29 (1973) 1–14.
10 T. SAWAI, T. YAMAKI, AND T. OHYA, *Agric. Biol. Chem.*, 40 (1976) 1293–1299.
11 S. J. MCWETHY AND P. A. HARTMAN, *Appl. Environ. Microbiol.*, 37 (1979) 1096–1102.
12 L.-H. WANG AND P. A. HARTMAN, *Appl. Environ. Microbiol.*, 31 (1976) 108–118.
13 A. AMEMURA, T. SUGIMOTO, AND T. HARADA, *J. Ferment. Technol.*, 52 (1974) 778–780.
14 M. YAMAMOTO AND K. HORIKOSHI, *J. Jpn. Soc. Starch Sci.*, 34 (1987) 304–307.
15 P. TRINDER, *Ann. Clin. Biochem.*, 6 (1969) 24–28.
16 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
17 H. FUWA, *J. Biochem. (Tokyo)*, 41 (1954) 583–603.
18 K. WALLENFELS, H. BENDER, AND J. R. RACHED, *Biochem. Biophys. Res. Commun.*, 22 (1966) 254–261.
19 K. YOKOBAYASHI, A. MISAKI, AND T. HARADA, *Biochim. Biophys. Acta*, 212 (1970) 458–469.
20 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404–427.
21 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406–4412.
22 T. AKINO, N. NAKAMURA, AND K. HORIKOSHI, *Appl. Microbiol. Biotechnol.*, 26 (1987) 323–327.
23 T. AKIBA AND K. HORIKOSHI, *Agric. Biol. Chem.*, 40 (1976) 1851–1856.
24 Y. IKURA AND K. HORIKOSHI, *Agric. Biol. Chem.*, 43 (1979) 1359–1360.