Biochimica et Biophysica Acta, 1036 (1990) 1–5
Elsevier

BBAGEN 23386

Purification and some properties of cyclodextrin-hydrolyzing enzyme from *Bacillus sphaericus*

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(Received 5 February 1990)
(Revised manuscript received 1 June 1990)

Key words: Cyclodextrin; Hydrolysis; Maltooligosaccharide; Polysaccharide; (*B. sphaericus*)

An intracellular cyclodextrin-hydrolyzing enzyme from *Bacillus sphaericus* E-244 isolated from soil was purified to a homogeneous state by means of Triton X-100 extraction, DEAE-Sepharose column chromatography, hydrophobic and molecular-sieve HPLC. The enzyme was estimated to have an M_r of 72000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis and 144000 by HPLC gel filtration on TSK gel G 3000 SW. It had a pH optimum of 8.0, and the enzyme, stable at 25°C and pH 5.5–9.5 for 24 h, was inactivated at 50°C for 10 min. The enzyme hydrolyzed β-cyclodextrin more effectively than linear maltooligosaccharides such as maltopentaose, maltohexaose and maltoheptaose or polysaccharides such as starch, amylopectin, amylose and pullulan.

Introduction

In general, the hydrolysis rate of cyclodextrins (CDs) by α-amylase is far slower than that of starch [1,2], and exotype amylases, such as glucoamylase and β-amylase, scarcely hydrolyze CDs [3,4]. On the other hand, cyclomaltodextrinase (EC 3.2.1.54) (CDase) can rapidly hydrolyze CDs [5,6], but has very little activity for polysaccharides such as starch and glycogen.

Up until now, CDases have been isolated from only two origins, one from *Bacillus macerans* [5,7] and the other from *Bacillus coagulans* [6]. In addition to the CDases, it has been reported that CDs are hydrolyzed faster than starch by only two enzymes, an α-amylase from *Pseudomonas* sp. [8] and a bacterial glucoamylase from *Flavobacterium* sp. [9]. However, these enzymes are not specific for CDs and there has been no report of an enzyme that can hydrolyze CDs most effectively among α-glucans.

Recently, we investigated bacteria which could produce maltooligosaccharides from CDs and isolated a soil bacterium that produced an intracellular enzyme which rapidly hydrolyzed β-CD. The present paper concerns the purification and some properties of the enzyme.

Maltooligosaccharides, especially maltoheptaose (G_7), or their derivatives have been utilized as a substrate for the assay of α-amylase activity in serum and urine [10]. The probability of usefulness of the CD-hydrolyzing enzyme is also shown in this paper.

Materials and Methods

Bactotryptone and yeast extract were purchased from Difco, and peptone from Kyokuto Seiyaku Kogyo, Japan. The β-CD was obtained from Nihon Shokuhin Kako, Japan, and CDs and pullulan from Hayashibara Biochemical Laboratories, Japan. Maltopentaose (G_5) and maltohexaose (G_6) were given by Seishin Pharmaceuticals, Japan. Potato starch was purchased from Sigma, and G_7, amylopectin, amylose and short-chain amylose (d.p. value 17) from Nacalai Tesque, Japan.

Cultures

The organism used for enzyme production was isolated from soil, identified and named *Bacillus sphaericus* E-244. Stock cultures of the organism were maintained on nutrient agar slants containing 2% bactotryptone, 1% yeast extract, 0.5% NaCl and 1.5% agar (pH 7.0). The production medium of the CD-hydrolyzing enzyme consisted of 1% β-CD, 1% peptone, 0.1% yeast

Abbreviations: CDs, cyclodextrins; CDase, cyclomaltodextrinase; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose; G_7, maltoheptaose; CGTase, cyclodextrin glucanotransferase; IR, infrared; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate.

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extract and 0.5% NaCl. The pH was adjusted to 7.0. For preculture, the organism was cultivated in a 500 ml Sakaguchi's flask, containing 100 ml of the medium, at 30°C for 24 h on a reciprocal shaker and 50 ml of the preculture was used as an inoculum for main culture. The main culture was carried out in a 3-liter jar fermentor (Iwashiya A-D-M, Japan) containing 2 liters of the medium at 30°C for 48 h. The aeration rate was 2 liters per min and the agitation rate was 350 rpm.

HPLC system

The HPLC system consisted of an HPLC pump, a Model CCPM equipped with a Refractive-Index detector, Model RI-8000 (Toso, Japan) and an ultraviolet-visible (UV-VIS) spectrophotometric detector Model UVIDEC 100-III (Japan Spectroscopics, Japan). Samples were injected automatically with an Auto-Sampler, Model AS-8000 (Toso, Japan), or manually with a Rheodyne Model 7125 sample injector (Rheodyne). A Model Chromatocorder II (System Instruments) was used for the determination of peak areas and retention times.

Assay of CD-hydrolyzing enzyme activity

The standard assay of CD-hydrolyzing enzyme was carried out with a reaction mixture containing 0.50 ml of enzyme solution in 100 mM phosphate buffer (pH 7.5) and 0.50 ml of 2% β-CD solution. After incubation at 40°C for 60 min, the reaction was stopped by boiling the mixture for 10 min. The amount of G_7 produced was determined by HPLC using authentic G_7 as a standard. The operation conditions of HPLC were as follows: column, TSK gel amide 80 (4 mm i.d. × 250 mm, Toso, Japan); solvent, water/acetonitrile (2 : 3, v/v); flow rate, 1 ml per min; column temperature, 35°C; and injection volume, 10 μl. Alternatively, for studies on substrate specificity and kinetics, enzyme activity was also determined through measurement of reducing sugar by the method of Somogyi-Nelson with glucose as a standard [11]. The reaction mixture containing 200 μl of enzyme solution in 100 mM phosphate buffer (pH 7.5) and 200 μl of 4 mM CDs and maltooligosaccharides or 0.2% polysaccharides substrate solution was incubated at 40°C for 60 min. After the reaction was stopped by boiling the mixture for 10 min, the amount of reducing sugar was determined. One unit of enzyme activity is defined as the amount which liberates 1 μmol of G_7 or adehyde groups as glucose from β-CD per min.

Purification of the CD-hydrolyzing enzyme

Cells of *B. sphaericus* E-244 were harvested from 8 liters of culture broth by centrifugation at 8000 × *g* and washed with 200 ml of 10 mM phosphate buffer (pH 7.0). The cells were suspended in 800 ml of the same buffer. Triton X-100 (16 ml) was added and the cell suspension was stirred at 25°C for 24 h. The cell debris was removed by centrifugation at 12000 × *g* for 20 min and the supernatant liquid was used as a crude enzyme preparation.

The crude enzyme solution (800 ml) was dialyzed against 10 mM phosphate buffer (pH 7.0) at 25°C for 16 h. Insoluble precipitate was removed by centrifugation at 12000 × *g* for 20 min. The supernatant solution was applied to a DEAE-Sepharose column (34 × 160 mm) equilibrated with 10 mM phosphate buffer (pH 7.0). Elution was obtained by a linear gradient of NaCl concentration from 0 to 0.5 M in the same buffer. The CD-hydrolyzing enzyme was eluted as a single peak at about 0.25 M of NaCl. The fractions with CD-hydrolyzing activity were combined, and Na_2SO_4 was added to the combined fractions up to the concentration of 1.0 M. The precipitates formed on standing for 60 min were removed by centrifugation at 12000 × *g* for 20 min. The supernatant liquid was applied to the TSK gel Ether 5PW column (21.5 mm i.d. × 150 mm; Toso, Japan) equilibrated with 100 mM phosphate buffer (pH 7.0) containing 1.0 M Na_2SO_4, and fractionated by the HPLC system. The hydrolase was eluted by decreasing the Na_2SO_4 concentration (1.0–0 M) at a rate of 5 ml per min and fractions of 5 ml were collected. The active fractions were pooled and concentrated to approx. 1.0 ml by ultrafiltration. 200 μl of the concentrated solution were applied to a TSK gel G 3000 SW column (7.15 mm i.d. × 600 mm × 2; Toso, Japan) equilibrated with 100 mM phosphate buffer (pH 7.0) containing 0.2 M NaCl, and 0.7 ml fractions were collected. The CD-hydrolyzing activity peaked at fraction numbers 40–41 and these fractions were used as a purified enzyme preparation to characterize its biochemical and enzymatic properties (Fig. 1). Purity of the protein was examined at each purification step by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [12]. An

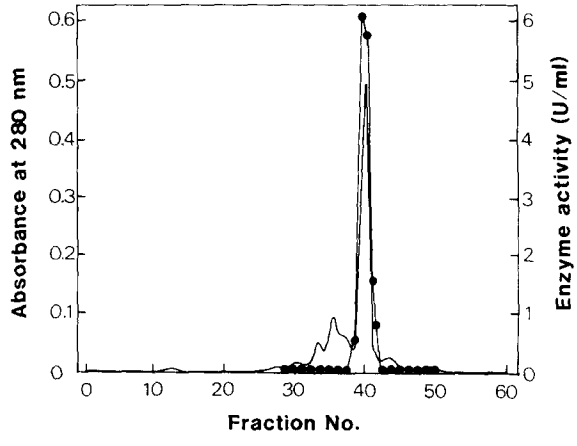

Fig. 1. Gel filtration of CD-hydrolyzing enzyme from *B. sphaericus* through TSK gel G 3000 SW. The experimental details are described in the text. Fractions of 0.7 ml were collected. Enzyme activity (●———●); and absorbance at 280 nm (———).

electrophoresis calibration kit (Boehringer-Mannheim Biochemica) was used to estimate molecular weight. Molecular weight was also determined with a TSK gel G 3000 SW column using MW-Marker for HPLC (Oriental Yeasts, Japan).

Protein concentration was estimated by the Coomassie blue method of Bradford using γ-globulin as a standard [13]. The protein contents in eluates from HPLC columns were expressed as the absorbance recorded automatically at 280 nm.

Enzymatic properties

The pH optimum and pH stability of the enzyme were studied in acetate buffer (pH 4.0–5.5), phosphate buffer (pH 5.5–8.0) and borate buffer (pH 7.5–10.0). To find optimal pH, the amount of G_7 was determined by the standard assay method using various 0.05 M buffer solutions. To determine pH stability, the purified enzyme (5.75 μg protein) in 100 mM of various buffer solutions (0.2 ml) was incubated at 25 °C for 24 h. A 0.1 ml portion of the enzyme solution was mixed with 0.4 ml of 100 mM phosphate buffer (pH 7.5) and 0.5 ml of 2% β-CD solution, and the residual activity was measured using the standard assay method.

The effects of metal ions on the CD-hydrolyzing enzyme were also examined to determine the activity after preincubation of the enzyme with 1 mM each of metal ions at 40 °C for 30 min. The initial velocities of the hydrolysis of various substrates were determined to measure reducing sugar produced. The K_m and V_{max} values were obtained by Lineweaver-Burk plot using 0.2 to 2.5 mM substrate and 0.13 μg purified enzyme.

Hydrolytic action on β-CD

The time-course of β-CD hydrolysis by the CD-hydrolyzing enzyme was obtained as follows. The reaction mixture containing 2 ml of enzyme solution in 10 mM phosphate buffer (pH 7.5) and 2 ml of 2% β-CD solution was incubated at 40 °C. 200 μl aliquots of the reaction mixture were withdrawn at regular intervals and boiled for 10 min. The denaturated protein was then removed by centrifugation at 8000 × *g* for 10 min, and the supernatant was analyzed by the standard assay method using authentic maltooligosaccharides and CDs as the standards. An infrared (IR) spectrum was taken with an IR spectrometer model JASCO A-202 (Japan Spectroscopics, Tokyo) and NMR analysis was done with an NMR spectrometer model JNM-FX 200 (JEOL, Japan).

Optical rotation study

To determine the configuration of the anomeric carbon atom of the products, the changes in the optical rotation were observed with time by the method of Kato et al. [8] on the reaction mixture of the CD-hydrolyzing enzyme with β-CD.

Results

Purification

Table I shows a summary of the purification of the CD-hydrolyzing enzyme from an 8 liter culture broth of *B. sphaericus*. The enzyme purified 69-fold to the original crude extract with 8% recovery. The purified enzyme found to be homogeneous as shown as a single band in SDS-polyacrylamide gel electrophoresis (Fig. 2).

General properties of CD-hydrolyzing enzyme

The molecular weight of the enzyme was estimated from the mobility on SDS-polyacrylamide gel electrophoresis to be 72000 and from the retention time of gel filtration using TSK gel G 3000 SW HPLC to be 144000. Based on these results, the enzyme was considered to be a homodimer. The enzyme showed maximal activity at pH 8.0 and was stable over the range of pH 4.5–9.5 at 25 °C. When it was tested at 30–80 °C in 0.1 M phosphate buffer (pH 7.5) for 15 min the enzyme was found to be stable at temperatures below 45 °C. The CD-hydrolyzing enzyme activity was almost completely inhibited by Hg^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} ions. With Ca^{2+}, Mg^{2+}, Li^{+}, Mn^{2+} and Fe^{3+} ions the

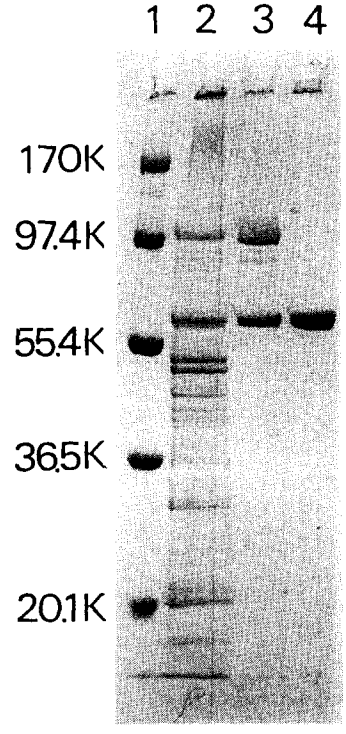

Fig. 2. SDS-polyacrylamide gel electrophoresis of proteins at different stages of purification. About 20 μg of each protein was treated with 15% 2-mercaptoethanol at 100 °C for 5 min and subjected on each column. The gel was stained with Coomassie brilliant blue R250. Lane 1, marker proteins; lane 2, active fraction after DEAE-Sepharose column chromatography; lane 3, active fraction after Ether 5PW HPLC; and lane 4, purified enzyme.

TABLE I

Summary of purification of CD-hydrolyzing enzyme from B. sphaericus

Procedure	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Crude extract	2135	522	0.24	100
Dialysis	2179	423	0.19	81
DEAE-Sepharose	373	300	0.80	57
TSK gel Ether 5PW HPLC	10.8	103	9.54	20
TSK gel G 3000 SW HPLC	2.6	43	16.61	8

enzyme activities were, respectively, 138, 111, 72, 59 and 42% of the control.

Substrate specificity

Table II shows reaction rates of the enzyme which splits the glycosidic bonds of various kinds of α-glucans. The CD-hydrolyzing enzyme can hydrolyze β-CD most rapidly among α-glucans. The rates of hydrolysis for polysaccharides (such as starch, amylopectin, amylose and pullulan) and for oligosaccharides (such as G_5, G_6 and G_7) were less than 10 and 70%, respectively, compared to that for β-CD.

TABLE II

Initial velocities of hydrolysis of various substrates by CD-hydrolyzing enzyme from B. sphaericus

Enzyme activities to split glycosidic bonds were measured by the Somogyi-Nelson method. Specific activity for β-cyclodextrin (16.6 units/mg) was taken as 100%.

Substrate	Concentration	Relative initial velocity (%)
β-Cyclodextrin	2 mM	100
α-Cyclodextrin	2 mM	44
Maltopentaose	2 mM	49
Maltohexaose	2 mM	43
Maltoheptaose	2 mM	66
Short-chain amylose	0.1%	44
Amylose	0.1%	3.7
Amylopectin	0.1%	0.7
Starch	0.1%	2.9
Pullulan	0.1%	1.5

TABLE III

Kinetic parameters for various substrates

Substrate concentration: 0.2 to 2.5 mM in 100 mM phosphate buffer (pH 7.5) at 40 °C.

Substrate	K_m (mM)	V_{max} (μmol/ml)	V_{max}/K_m
β-Cyclodextrin	0.38	0.110	0.290
α-Cyclodextrin	0.71	0.074	0.104
γ-Cyclodextrin	1.43	0.039	0.027
Maltotetraose	1.54	0.067	0.044
Maltopentaose	0.95	0.073	0.077
Maltohexaose	1.11	0.075	0.068
Maltoheptaose	0.69	0.060	0.087

The K_m and V_{max} values of the enzyme for various substrates were shown in Table III. The K_m value for β-CD was the smalelst among linear and cyclic maltooligosaccharides. The K_m/V_{max} value for β-CD was the largest among them, and was about 3-times larger than that for G_7. The K_m/V_{max} value for α-CD was larger than that for G_6.

Hydrolytic action for β-CD

Fig. 3A and B illustrate the time-course of the hydrolysis of β-CD by the CD-hydrolyzing enzyme. The product from β-CD as only G_7 up to 20% hydrolysis of β-CD. When the hydrolysis of β-CD reached about 70%, the ratio of G_7 to total sugars in the reaction mixture was greater than 50%. The G_7 then gradually degraded to various linear maltooligosaccharides of smaller molecular sizes (such as G_6, G_5, G_4, G_3 and so on). The main product from β-CD, G_7 assumed by the

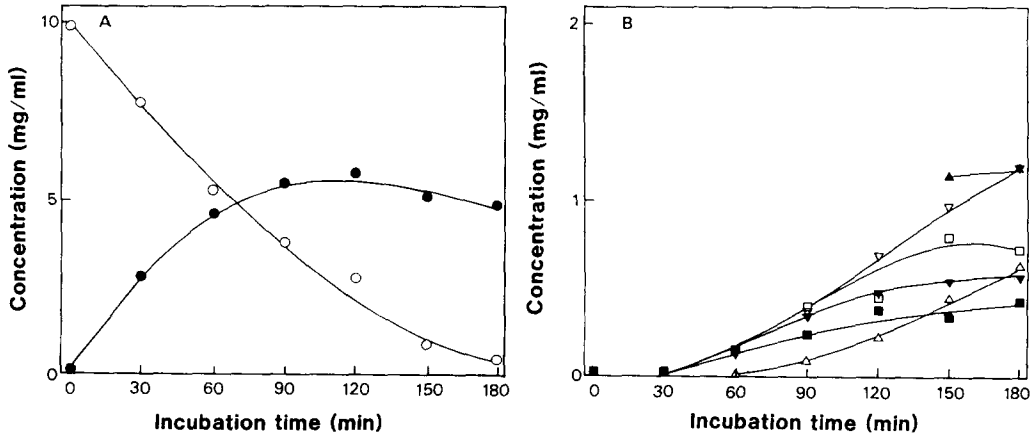

Fig. 3. Time-course of hydrolytic action for β-cyclodextrin by CD-hydrolyzing enzyme from *B. sphaericus*. Reaction conditions were as described in the text. (A) β-CD (○) and maltoheptaose (●). (B) Glucose + maltose (△), maltotriose (▽), maltotetraose (▲), maltopentaose (■), maltohexaose (□) and α-CD (▼).

HPLC analysis, was further identified as G_7 by IR and NMR analyses.

Optical-rotation study

It was found by optical rotation study that the CD-hydrolyzing enzyme produced an α-anomer from β-CD.

Discussion

CD-hydrolyzing enzyme was isolated from a Triton X-100 extracts of *B. sphaericus* cells, and purified approx. 70-fold with a yield of 8%. The apparent purity of the enzyme was demonstrated by SDS-polyacrylamide gel electrophoresis. It hydrolyzed CD and maltooligosaccharides specifically, but hydrolyzed starch, amylose, amylopectin and pullulan to a lesser extent.

CD-hydrolyzing enzymes which had ever been reported were α-amylase from *Pseudomonas* sp. [8], glucoamylase from *Flavobacterium* sp. [9] and CDases from *B. macerans* and *B. coagulans* [4,5]. Although the K_m values of α-amylase from *Pseudomonas* sp. and glucoamylase from *Flavobacterium* sp. for β-CD were not determined, those of CDases from *B. macerans* and *B. coagulans* for β-CD have been reported to be 2.65 and 2.8 mM, respectively [5,6]. On the other hand, the K_m value of the CD-hydrolyzing enzyme from *B. sphaericus* for β-CD was 0.38 mM, which indicates that our enzyme has far higher affinity for β-CD than other CDases. The enzyme activity was maximum at about pH 8.0 on the alkaline side, while the others had optimum pHs at pH 5.5–6.5 on the acidic side [5,6,8,9].

The enzyme consisted of two 72 kDa subunits and its whole molecular mass was calculated to be 144 kDa by the gel filtration method. De Pinto and Campbell [5] reported that the $S_{20,w}$ value of CDase from *B. macerans* was 23S, but its molecular mass was not reported. The molecular masses of CDase from *B. coagulans* and α-amylase from *Pseudomonas* sp. were estimated to be 64 and 96 kDa, respectively, by SDS polyacrylamide gel electrophoresis [6,7]. This indicates that the above enzymes are different from our enzyme.

CD glucanotransferase (CGTase) has been discovered in many kinds of bacteria which catalyzed the conversion of starch to CD by intramolecular transglycosylation (cyclization reaction) [14]. This enzyme also catalyzes the hydrolysis of CDs and α-1,4-glucans, but the cyclization reaction is much stronger than the hydrolytic reaction. We examined whether or not CD-hydrolyzing enzyme from *B. sphaericus* has cyclization activity by incubation with starch, amylose or amylopectin for 1 h using 0.21 units of the enzyme. We could not detect any kind of CD in the incubation mixture, indicating that our enzyme does not have CGTase activity. Comparison of additional biochemical properties among CGTase or other CD-hydrolyzing enzyme is difficult because of limited information. Nevertheless, some biochemical characteristics of the CD-hydrolyzing enzyme from *B. sphaericus* were similar to those established for the other CDases.

Application of the CD-hydrolyzing enzyme discovered by us provides a means for producing a large amount of G_7 from β-CD. The use of already reported CDases, α-amylase and CGTases is not appropriate for this purpose, because G_7 produced from β-CD was further hydrolyzed by these enzymes to smaller oligosaccharides simultaneously. The hydrolysis of β-CD by the CD-hydrolyzing enzyme from *B. sphaericus* resulted in the accumulation of G_7 for up to 60 min incubation under the condition used, and the yield of G_7 from β-CD was about 50% at that time. The accumulation of G_7 might be explained by the kinetic studies. Since this enzyme had the highest catalytic efficiency to β-CD among α-glucans, it could rapidly produce G_7 from β-CD. However, the hydrolysis rate of G_7 to the smaller oligosaccharides was far smaller than that of β-CD to G_7. Therefore, this enzyme is useful for mass production of G_7 from β-CD.

Acknowlegdements

The authors express their sincere thanks to Dr. S. Nasuno for his preparation of the manuscript and Dr. N. Saito for significant advice. We would also like to thank Ms. A. Nasu and Mr. K. Kasai for the technical performance of NMR and IR analyses, and Ms. T. Okubo for her technical assistance.

References

1 Ohnishi, M. (1971) J. Biochem. (Tokyo) 69, 181–189.
2 Suetsugu, N., Koyama, S., Takeo, K. and Kuge, T. (1974) J. Biochem. (Tokyo) 76, 57–63.
3 Kobayashi, S., Kainuma, K. and Suzuki, S. (1973) Proceedings of the Symposium on Amylase (in Japanese) 8, 29–36.
4 Thoma, J.A. and Koshland, D.E. (1960) J. Am. Chem. Soc. 82, 3329–3333.
5 DePinto, J.A. and Campbell, L.L. (1968) Biochemistry 7, 121–125.
6 Kitahata, S., Taniguchi, M., Beltran, S.D., Sugimoto, T. and Okada, S. (1983) Agric. Biol. Chem. 47, 1441–1447.
7 DePinto, J.A. and Campbell, L.L. (1968) Arch. Biochem. Biophys. 125, 253–258.
8 Kato, K., Sugimoto, T., Amemura, A. and Harada, T. (1975) Biochim. Biophys. Acta 391, 96–108.
9 Bender, H. (1981) Eur. J. Biochem. 115, 287–291.
10 Henkel, E., Morich, S. and Henkel, R. (1984) J. Clin. Chem. Clin. Biochem. 22, 489–495.
11 Somogyi, M. (1952) J. Biol. Chem. 195, 19–23.
12 Laemmli, U.K. (1970) Nature 227, 680–685.
13 Bradford, M. (1976) Anal. Biochem. 72, 248–254.
14 Kitahata, S. (1988) in Handbook of Amylases and Related Enzymes (The Amylase Research Society of Japan, ed.), pp. 154–164, Pergamon Press, Tokyo.