Preparation and Some Properties of Active Monomer of Sweet Potato β -Amylase

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Received October 9, 1989

Sweet potato β -amylase was divided into two active fractions (named F-A and F-B) by modification with periodate-oxidized maltohexaose at pH 9.7. F-A and F-B isolated by exclusion chromatography using Sephadex G-200 corresponded to a pentamer of the enzyme with molecular weight of 31.5×10^4 and a monomer with molecular weight of 6.4×10^4 , respectively. The contents of carbohydrate of the modified enzymes were 9.7% for F-A and 9.3% for F-B. The specific activities of F-A and F-B were 2,059 and 1,129 units/mg of protein and were reduced to 82% and 45% of that of the original enzyme, respectively. The modification of the enzyme with the oxidized maltohexaose was due to formation of a Schiff base between ϵ -NH₂ groups of lysines of the enzyme protein and CHO groups of the oxidized maltohexaose.

Since sweet potato β -amylase (α -1,4-glucan maltohydrolase EC 3.2.1.2) was first isolated as a crystalline form by Balls *et al.* in 1948,¹⁾ the physicochemical and enzymatic properties have been studied in detail.²⁾ Also, the enzyme is known as a tetrameric enzyme with molecular weight of 21.5×10^4 which consists of four identical subunits,³⁾ though most of the β -amylases are monomeric enzymes.^{4,5)} Recently, the molecular weight of the subunit of the enzyme was exactly calculated to be 55,707 based on its amino acid sequence (496 residues).⁶⁾ The relationship between subunit structure and catalytic function of the enzyme is not known.

More recently, we have published how the existence of the active monomer of sweet potato β -amylase was elucidated by the modification of the enzyme with periodate-oxidized soluble starch under alkaline conditions in the absence of any reducing agent.⁷⁾

This paper deals with the modification of sweet potato β -amylase with periodate-oxidized maltohexaose and some properties of the modified enzyme by the modification.

Materials and Methods

1. *Sweet potato β -amylase.* Sweet potato β -amylase was purified from the juice of mashed sweet potato (a variety of Japanese Kokei 14) by our previous method⁸⁾ with a slight modification to increase the purity and the yield of the enzyme. The juice was first heated at 60°C for 10 min and cooled down to 20°C to stabilize the β -amylase handling in acetone at room temperature,⁹⁾ and centrifuged at 10,000 rpm for 10 min. Then, acetone was added to the supernatant at a concentration of 50%. The resulting precipitate was collected by centrifuge (4,000 rpm, 5 min) and dissolved in a proper amount of water. The β -amylase was precipitated again with acetone of a concentration of 45% and the resulting precipitate was collected and dialyzed against a 0.01 M Tris-HCl solution of pH 6.8. The dialyzed solution was then put on a DEAE Sephadex A-50 column (4.2 $\times$ 48 cm) equilibrated with the same solution. After washing the column with a large amount of the same solution, the enzyme was eluted with a linear gradient of sodium chloride (0.1-0.5 M). The active fractions were combined and concentrated. The concentrate was then chromatographed on a Sephadex G-200 column (3.9 $\times$ 95 cm) in 0.05 M acetate buffer (pH 5.5) containing 0.15 M NaCl. The active fractions were collected and concentrated by addition of an equal volume of acetone. The crystallization of the enzyme was done twice from distilled water. The purified β -amylase was homogeneous and showed only alanine as the NH₂-terminal

amino acid. The specific activity of the recrystallized enzyme was 2,500 units/mg and the enzyme preparation did not show both activities of α -amylase and α -glucosidase when tested by a high performance liquid chromatography (described below) of the reaction products.

2. *Oxidized maltooligosaccharides.* Two grams of maltooligosaccharides were dissolved in 100 ml of a 0.2 M NaIO₄ solution and oxidized with gently stirring at 4°C for 40 min, and 10 ml of ethylene glycol was then added to the reaction mixture to stop the oxidation. Then the reaction mixture was chromatographed on a column of Sephadex G-25 (1.9 × 150 cm) in distilled water. The oxidized maltooligosaccharides were assayed both by measuring absorbance at 280 nm for its aldehyde groups and by the phenol-H₂SO₄ method¹⁰⁾ for total sugar. The purified oxidized maltooligosaccharides were lyophilized. The oxidized soluble starch was prepared by our method, reported previously.⁷⁾ The CHO groups of the maltooligosaccharides measured by the method of Somogyi-Nelson¹¹⁾ were 1.06 mmol/g for maltose, 2.20 mmol/g for maltotetraose, 2.33 mmol/g for maltohexaose, and 2.05 mmol/g for soluble starch, respectively.

3. *Chemicals.* Maltooligosaccharides were kindly supplied from Nihon Shokuhin Kako Co., Ltd. Soluble starch was purchased from Kokusan Chemical Co., glyceraldehyde from Wako Pure Chemicals Industries Ltd., a protein kit for measurement of molecular weight from Bio Rad Chemicals, and thyroglobulin, ferritin, catalase, and aldolase from Pharmacia Fine Chemicals. Other chemicals were of analytical grade.

4. *Enzyme assay and protein measurement.* For enzyme assay, the enzyme was diluted with 0.05 M acetate buffer, pH 4.8, containing 0.04% Triton X-100 to prevent denaturation of the enzyme.¹²⁾ A sample of 0.2 ml of the enzyme solution was added to 0.2 ml of 2% soluble starch in 0.05 M acetate buffer containing 0.04% Triton X-100, pH 4.8. After 10 min of incubation at 37°C, the maltose formed in the reaction mixture was measured by the method of Somogyi-Nelson.¹¹⁾ One unit of the enzyme activity was defined as the amount of enzyme which produced 1 μ mol of maltose per min under these conditions.

Protein was measured spectrophotometrically on a Hitachi Spectrophotometer Model 139 by assuming that the $E_{280\text{ nm}}^{1\%}$ of the enzyme was 17.1.¹³⁾

5. *High performance liquid chromatography (HPLC).* HPLC was done for analyses of purity and the molecular size of the modified and original enzymes on Shimadzu model LC 6A system using a SynChropak GPC 100 column (0.78 × 30 cm) and 0.1 M K-phosphate buffer, pH 6.8, as a developing solution at a flow rate of 0.3 ml/min or 0.5 ml/min, and detection of the enzymes were done on a Shimadzu UV/visible monitor Model SPD 6AV. Also,

sugar analysis was done by HPLC using Shimpak SCR 101 N (0.79 × 30 cm) in distilled water as a eluent at a flow rate of 1.0 ml/min, and the sugars were monitored with a Knauer differential refractometer.

6. *Sodium dodecyl sulfate-linear gradient polyacrylamide gel electrophoresis (SDS-PAGE).* SDS-PAGE was done on a gel plate (13.5 × 13.5 cm, 0.1 cm thickness) of a linear gradient concentration of polyacrylamide from 5% to 20% in the presence of 0.1% SDS in a Tris buffer system of pH 8.8 at 40 mA for 3 hr.¹⁴⁾ Samples were heated with 1% SDS at 100°C for 3 min before electrophoresis. Three μ g of the sample was used for protein staining with Coomassie brilliant blue (CBB) and 24 μ g for carbohydrate staining with periodic acid-Schiff (PAS).¹⁵⁾

7. *Analyses of amino acid composition, NH₂-terminal amino acid, and ϵ -NH₂ of lysine.* The amino acid composition of the original enzyme and the modified enzyme with the oxidized maltohexaose were analyzed by the method of Spackman *et al.*¹⁶⁾ on a Hitachi model 835 amino acid autoanalyzer. Samples were hydrolyzed with 6 N HCl in an evacuated and sealed tube at 105°C for 24, 48, and 72 hr before analysis. Terminal-NH₂ and ϵ -NH₂ group of lysine of the original and modified enzyme were measured by the dinitrophenylation method of Sanger.¹⁷⁾ Dinitrophenylated (DNP)-enzymes were hydrolyzed with 6 N HCl at 105°C for 16 hr. The ether-soluble and water-soluble DNP-amino acids were identified by thin layer chromatography on a silica gel plate (19.5 × 19.5 cm, 0.1 cm thickness) using toluene-pyridine-ethylene chlorohydrin-0.8 N ammonia (10:3:6:6) and *n*-propanol-34% ammonia (7:3) as developing solvents, respectively. The DNP-amino acids were measured spectrophotometrically after extraction from the plate.

Results

1. *Modification of sweet potato β -amylase with various oxidized maltooligosaccharides*

The modification of sweet potato β -amylase with three kinds of maltooligosaccharides as well as the oxidized soluble starch were first investigated for preparation of the active monomer. As shown in Fig. 1, three peaks besides a peak of the original enzyme appeared newly on HPLC in a certain ratio in every case except for the modification with glyceraldehyde. The first peak from the left of Fig. 1 had no activity, while both the second and fourth peaks had the activity, which corresponded to the modified pentamer and monomer of the enzyme, respectively, as described below.

Fig. 1. HPLC Patterns of Sweet Potato β -Amylase Modified with Several Oxidized Maltooligosaccharides and Soluble Starch.

Each reaction mixture contained 50 μ g of the enzyme and 500 μ g of the oxidized carbohydrate in 0.1 ml of 0.15 M H_3BO_3 -KCl- Na_2CO_3 buffer, pH 9.7, and was incubated at 37°C for 10 min, and then chromatographed by HPLC. Modified with: 1, oxidized soluble starch; 2, oxidized maltohexaose; 3, oxidized maltotetraose; 4, oxidized maltose; 5, glyceraldehyde; and 6, original enzyme.

These results show that effective molecular sizes of oxidized glucans for obtaining the active monomer were at least bigger than that of maltohexaose. Also, when the modification was done at neutral pH with various oxidized glucans, only the pentamer was detected. We therefore tried to prepare the active monomer of the enzyme by modification with the oxidized maltohexaose at pH 9.7.

2. Preparation of an active monomer of sweet potato β -amylase

The modification of sweet potato β -amylase with the oxidized maltohexaose was done by

Fig. 2. Exclusion Chromatography of Sweet Potato β -Amylase Modified with Oxidized Maltohexaose on a Column of Sephadex G-200.

Thirty mg of sweet potato β -amylase was incubated with 120 mg of the oxidized maltohexaose in 10 ml of 0.15 M H_3BO_3 -KCl- Na_2CO_3 buffer, pH 9.7, at 37°C for 10 min. Then the pH of the mixture was adjusted to 7.5 by addition of a 1 M NaBH_4 -HCl solution, pH 4.5. After this was dialyzed against 0.1 M NaBH_4 -HCl buffer, pH 7.5, at 4°C for 24 hr, the dialyzed solution was put on the column (3.5×104 cm) in 0.1 M K-phosphate buffer containing 0.15 M NaCl of pH 6.8. $\circ$, absorbance at 280 nm; $\bullet$, activity; $\triangle$, carbohydrate content.

the procedures described in the legend of Fig. 2. The modified preparation was chromatographed on a column of Sephadex G-200, as shown in Fig. 2, separating it into three fractions. The first peak from the left of the figure did not show any activity and was considered to be denatured by exposure to the alkaline buffer of pH 9.7 during the modification. The second and third peaks (F-A and F-B) were each collected and pooled. Both fractions were active and were homogeneous by HPLC (Fig. 3). Specific activity of F-A and F-B were 2,059 and 1,129 units/mg protein, respectively.

3. Molecular weights of F-A and F-B

The molecular weights of both fractions were estimated by exclusion chromatography on Sephadex G-200. As shown in Fig. 4, the molecular weight of F-A was estimated to be 31.5×10^4 and it was bigger than the original

Fig. 3. HPLC Patterns of the Modified Enzymes and the Original Enzyme.

1, reaction mixture before the exclusion chromatography on Sephadex G-200; 2, F-A; 3, F-B; 4, original enzyme.

Fig. 4. Estimation of Molecular Weights of F-A and F-B by Exclusion Chromatography by Sephadex G-200.

The chromatographic conditions were the same as those described in Fig. 2. ○, F-A; ●, F-B; □, original enzyme; △, markers: 1, thyroglobulin (669,000), 2, ferritin (440,000), 3, catalase (232,000), 4, aldolase (152,000), 5, bovine serum albumin (67,000).

enzyme with molecular weight of 21.5×10^4 and corresponded to the molecular weight of the pentamer. On the other hand, the molecular weight of F-B was estimated to be 6.7×10^4 .

The molecular weights of the two fractions were also investigated by SDS-PAGE. As

Fig. 5. SDS-PAGE of F-A, F-B and Original Enzyme.

A, F-A stained with PAS; B, F-B stained with PAS; C, F-A stained with CBB; D, F-B stained with CBB; E, original enzyme stained with CBB; F, markers: from the top to the bottom: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and egg lysozyme.

shown in Fig. 5, both the molecular weight of F-A and F-B were estimated to be 6.4×10^4 , which were 1.15 times bigger than that of the monomer of the original enzyme whose molecular weight was 5.55×10^4 .

4. Carbohydrate contents of F-A and F-B

The carbohydrate contents of F-A and F-B measured by the phenol- H_2SO_4 method¹⁰⁾ were 9.7% and 9.3%, respectively, while the original enzyme contained no carbohydrate.

Furthermore, the gel was stained by the method of PAS¹⁵⁾ after electrophoresis to check whether or not the modification of sweet potato β -amylase with the oxidized maltohexaose was done covalently. As shown in Fig. 5, the mobility of the band stained by PAS was found to coincide with that of band stained by CBB in both cases, though the original enzyme was stained by CBB but not PAS. This suggests that the modification was done by covalent bonds.

5. Analyses of NH_2 -terminal amino acid, ϵ - NH_2 of lysine and amino acid composition of F-A and F-B

Both NH_2 -terminal amino acids of F-A and F-B were identified as alanine of which the

Fig. 6. Thermal and pH Stabilities of F-A and F-B.

A, pH stabilities. After 10 μ g of the enzyme in 1 ml of 0.1 M Britton–Robinson buffer was incubated at 37°C for 2 hr, it was diluted with 0.05 M acetate buffer, pH 4.8, containing 0.04% Triton X-100 before assay of the remaining activities. B, Thermal stabilities. Ten μ g of the enzyme in 1 ml of 0.1 M K-phosphate buffer, pH 6.8, was incubated for 1 hr and then diluted with the same solution as A before assay the remaining activities. ○, F-A; ●, F-B; △, original enzyme.

contents were 0.172 and 0.155 mol/mol of monomer (5.55×10^4), respectively, while it was 0.574 mol/mol of monomer for the original enzyme by the dinitrophenylation method.¹⁷⁾ From these results the α -NH₂ groups of the termini reacted with the CHO groups were calculated to be 70% for F-A and 73% for F-B. On the other hand, contents of the ϵ -DNP lysines of F-A and F-B were 6.45 and 7.02 mol/mol of monomer, respectively, while that of the original enzyme was 9.65 mol/mol of monomer, meaning that ϵ -NH₂ groups of lysine modified with oxidized maltohexaose were 33% for F-A and 26% for F-B.

From the results of amino acid analysis, the contents of the lysine of F-A and F-B were 20.5 and 20.3 mol/mol of monomer, respectively, while it was 27.3 mol/mol of the monomer for the original enzyme. These results mean that the total ϵ -NH₂ groups of lysine modified with CHO groups were 25% for F-A and 26% for F-B. These results agreed with the result obtained by the dinitrophenylation method described above.

6. Thermal- and pH-stabilities of F-A and F-B

The thermal- and pH-stabilities of both fractions were investigated compared with the original enzyme. As shown in Fig. 6B, the thermal stability of F-A was almost the same as the original enzyme, while F-B was much

less stable than the original enzyme at temperatures higher than 20°C. Also, F-A was as stable as the original enzyme in alkaline and acidic pH (Fig. 6A).

Discussion

Interestingly, sweet potato β -amylase seems to be unique in being a tetrameric enzyme consisting of four identical polypeptide chain subunits, though most plant and microbial β -amylases are monomeric.^{4,5)} Sweet potato β -amylase dissociates into monomers and loses the activity at high dilution.¹⁸⁾ This study was done to explain the relationship between subunit structure and catalytic function of the enzyme, and the result was that an active monomer of the β -amylase, named F-B in this paper, could be prepared by modification with periodate-oxidized maltooligosaccharides at pH 9.7 without any reducing agent.

The modification of sweet potato β -amylase to the monomer (F-B) and pentamer (F-A) with the oxidized maltohexaose are considered to form a Schiff base between ϵ -amino groups of lysines of the enzyme protein and aldehyde groups of the oxidized maltohexaose, from the results of the amino acid- and dinitrophenylation analyses. However, the reason for association into the pentamer is not resolved and remains for future study.

Essentially, an active monomer of sweet potato β -amylase may exist, because besides the results of these studies, we obtained a preliminary result in which the active monomer was found to exist by analysis of HPLC when the enzyme was treated with 6 M urea without any reducing agent, but it was very unstable and aggregated immediately to lose the activity. The fact suggests that the modification with the oxidized maltohexaose can stabilize the active monomer of the enzyme. Therefore, the tetrameric form of the enzyme is a most stable one and exists in nature, and the subunit structure of the enzyme may have no relation to catalytic function directly. In addition, besides the result of SDS-PAGE, the phenomenon that the enzyme dissociated into monomer in the absence of any reducing agent suggests that the enzyme does not have any S-S linkage for formation of the tetrameric form.

Previously, we showed that the active monomer of sweet potato β -amylase was prepared by modification using periodate oxidized soluble starch.⁷⁾ The oxidized soluble starch as well as the oxidized maltohexaose are considered to be effective in preparation of the active monomer. If the isolation of the active monomer of sweet potato β -amylase succeeded by reason of increasing the thermo- and(or) pH-stability, the oxidized soluble starch may be used industrially for conversion of thermo- and(or) pH-unstable enzymes to stable enzymes. We are investigating the modification of sweet potato β -amylase with the periodate-oxidized glucan in the presence of α -cyclodextrin, a competitive inhibitor of β -amylase,¹⁹⁾ which protects the active center of the enzyme during modification. The improvement of the

modification method will be published elsewhere.

References

- 1) A. K. Balls, M. K. Walden and R. R. Thompson, *J. Biol. Chem.*, **173**, 9 (1948).
- 2) Y. Takeda, in "Handbook of Amylases and Related Enzymes," ed. by The Amylase Research Society of Japan, Pergamon Press, Oxford, 1988, pp. 89-91.
- 3) J. A. Thoma, D. E. Koshland, Jr., J. Rucia and R. Baldwin, *Biochem. Biophys. Res. Commun.*, **12**, 184 (1963).
- 4) J. A. Thoma, J. E. Spradlin and S. Dygert, in "The Enzymes," Vol. 5, ed. by P. D. Boyers, Academic Press, New York and London, 1971, pp. 115-189.
- 5) R. Shinke, in "Handbook of Amylases and Related Enzymes," ed. by The Amylase Research Society of Japan, Pergamon Press, Oxford, 1988, pp. 81-83.
- 6) H. Toda, *Denpun Kagaku*, **36**, 87 (1989).
- 7) Y. G. Ann, M. Iizuka, N. Minamiura and T. Yamamoto, *Agric. Biol. Chem.*, **53**, 3109 (1989).
- 8) J. P. Kim, Y. G. Ann and W. M. Shim, *Korean Biochem. J.*, **18**, 290 (1985).
- 9) S. Nakayama and S. Amagase, *J. Biochem.*, **54**, 375 (1963).
- 10) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 11) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
- 12) Y. Takeda and S. Hizukuri, *Biochim. Biophys. Acta*, **268**, 175 (1972).
- 13) S. Englard and T. P. Singer, *J. Biol. Chem.*, **187**, 213 (1950).
- 14) P. T. Matsudaira and D. R. Burgess, *Anal. Biochem.*, **87**, 386 (1978).
- 15) R. M. Zacharius, T. E. Zell, J. H. Morrison and J. J. Woodlock, *Anal. Biochem.*, **30**, 148 (1969).
- 16) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 190 (1958).
- 17) F. Sanger and E. O. P. Thompson, *Biochem. J.*, **53**, 353 (1953).
- 18) P. Bernfeld, B. J. Berkeley and R. E. Bieber, *Arch. Biochem. Biophys.*, **111**, 31 (1965).
- 19) J. A. Thoma and D. E. Koshland, Jr., *J. Am. Chem. Soc.*, **82**, 3329 (1960).