An Isoelectric Separation of Soybean β-Amylase Isoforms and Their Enzymic Characteristics

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Authentic soybean β-amylase preparation, purified to homogeneity as judged by SDS-PAGE by using an affinity purification step, was composed of four pI-differing isoforms. By chromatofocusing, these isoforms were separated into three fractions, designated as fractions 1–3 in the order of elution. Fraction 1 contained two isoforms having the same molecular mass (55,989 Da), as measured by mass spectrometric analysis, with different pIs, 5.32 (Isoform I) and 5.22 (Isoform II). Fraction 2 showed a single isoform having a molecular mass of 55,994 Da and having a pI of 5.09. This component, named Isoform III, existed rather in excess in a mixture of the authentic enzyme isoforms. The remainder (fraction 3) also contained a single component (Isoform IV) which has a molecular mass of 56,310 Da with a pI of 4.97. Chemical analyses indicated that the N-termini and the C-terminal triptides of four pI-separated isoforms mentioned are similar to one another, and are blocked and are NH$_2$-Val-Asp-Gly-COOH, respectively. Moreover, enzymic properties involving specific activity and the value of kcat/Km for the above three fractions are almost the same, and also agreed completely with those of an unfractionated authentic β-amylase preparation.

Key words: soybean β-amylase; pI-heterogeneity; kinetic analyses; chromatofocusing; mass spectrometric analyses

β-amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2) catalyzes the successive liberation of maltose with the anomic configuration from the non-reducing ends of α-1,4-D-glucopyranosyl polymers such as starch and glycogen. This enzyme occurs in plants and in certain bacteria. Numerous studies have been reported on multiplicity of the enzyme from barley, wheat, rice, pea, rye, soybean, and Bacillus polymyxa, but most of the cases dealt with isoforms of the enzyme, in terms of molecular mass, which may be caused by in vivo proteolytic modifications or during the purification procedure. In case of soybean β-amylase, seven pI-different isoforms were observed, and three of them were isolated by a multiple column chromatography using a large amount (more than 20 kg) of soybean flour as a starting material. Those isoforms differed from another in Km and heat-stability.

Previously, we established a considerable purification procedure for soybean β-amylase, which includes an affinity-chromatographic step using α-cyclomaltoolhaxose-immobilized Sepharose 6B in the presence of ammonium sulfate. We have also established an E. coli-expression system of β-amylase cDNA derived from a cotyledon cDNA library of midmatured soybeans. The heat-stability and kinetic constants, such as V$_{max}$ and K$_m$ of the recombinant β-amylase differed greatly from those of authentic enzyme from soybean seeds. In attempting to define the causes that affect the quality of the recombinant enzyme, a possible point to consider is the existence of mRNA species coded for a β-amylase isoform having a low enzymic quality compared to that of authentic enzyme, naturally occurring as a mixture of several pI-differing isoforms.

Based on the foregoing, this study was aimed at characterizing the physicochemical and enzymic properties of pI-different isoforms separated from highly purified authentic β-amylase preparation from soybean [Glycine max (L., Merr.)].

Materials and Methods

Materials. Freshly milled soybean flour from mature seeds was defatted by n-hexane at about 65°C (kindly provided by Fuji Seiyu Company, Japan) and stored at 4°C until used. CM-Toyopearl 650 M was obtained from Tosoh Corporation, Japan and α-cyclomaltoolhexose (α-CD) was from Nihon Shokuhin Kako Corporation, Japan. Epoxy-activated Sepharose 6B was purchased from Pharmacia Biotech AB, Sweden. All inorganic chemicals were from Wako Pure Chemical Corporation, Japan.

Purification of authentic β-amylase from soybean flour. Crude enzyme protein was extracted by stirring 20 g of the defatted flour in 200 ml of 50 mM acetate buffer, pH 5.7, containing 3.5 mM 2-mercaptoethanol for 2 h at 4°C. After centrifugation, the supernatant was fractionated by adding solid ammonium sulfate and the precipitate obtained with 45–75% saturated ammonium sulfate was resuspended and then dialyzed against 10 mM acetate buffer, pH 5.0, containing 3.5 mM 2-mercaptoethanol overnight. Insoluble materials were removed by centrifugation and the resulting supernatant was put on a column (3.0×30 cm) of CM-Toyopearl 650 M equilibrated with 10 mM acetate buffer, pH 5.0,
containing 3.5 mM 2-mercaptoethanol and eluted by the same buffer with a linear 0–0.5 M gradient of sodium chloride at a flow rate of 2 ml/min. Fractions having an intensive β-amylase activity were pooled and then dialyzed against 50 mM acetate buffer, pH 5.7, containing 3.5 mM 2-mercaptoethanol in the presence of 1 mM ammonium sulfate. This protein sample was put on a column (1.0 × 10 cm) of α-cyclomaltotetraose-immobilized Sepharose 6B at a flow rate of 0.2 ml/min. After washing out unbound proteins with the same buffer, the protein bound to the affinity column was eluted with the acetate buffer in the absence of ammonium sulfate.14 As a control, recombinant (non-mutant II) β-amylase, produced in E. coli, was also purified by the same method.15

SDS-PAGE. Run of protein preparations was done on a 10–20% linear gradient acrylamide gel (Daichi Pure Chemicals Co., Japan) under reducing conditions according to the manufacturer’s instructions. After electrophoresis, protein was stained with Coomassie brilliant blue R-250. Pharmacia low-molecular-mass markers were used.

Isoelectric focusing on Ampholine PAGplate and on Immobiline gel. To measure the pI of individual isoforms of the authentic enzyme preparation, polyacrylamide gel isoelectric focusing was done using Ampholine PAGplate (Ampholine PAGplate pH 3.5–9.5, Pharmacia Biotech AB, Sweden), and then using Immobiline gel (Immobiline Dryplate pH 4–7, Pharmacia Biotech AB, Sweden) according to the manufacturer’s procedure. The relative pI for each isoform was measured with a commercial pI-calibration protein kit ranging from 2.5 to 6.5 (Pharmacia Biotech AB, Sweden).

Separation of pI-different β-amylase isoforms by chromatofocusing. For preparative separation of the pI-different isoforms, the purified enzyme protein (about 1.2 mg) was exhaustively dialyzed against distilled water containing 1 mM 2-mercaptoethanol overnight. The desalted protein solution was mixed with histidine-HCl buffer, pH 6.2, at a final concentration of 25 mM according to the manufacturer’s instructions, and then put on a Mono P column (Mono P HR 5/20, Pharmacia Biotech AB, Sweden) pre-equilibrated in the same histidine buffer. After washing with the buffer, chromatofocusing separation was done by a linear pH gradient in the range 6.2–4.0 using Polybuffer 74-HCl, pH 4.0 (Pharmacia Biotech AB, Sweden), and fractionation (each tube contained about 500 μl) was done beyond 120 tubes.

Assay method. The activity of β-amylase preparations was assayed as described previously.13 The standard reaction mixture (1 ml) was composed of 0.5 ml of 0.5% (w/v) soluble starch (Merck Co., USA) treated with sodium borohydride,10 0.4 ml of 50 mM sodium acetate buffer, pH 5.7, containing bovine serum albumin at 0.4 mg/ml, and 0.1 ml of the enzyme solution. Sodium borohydride treatment reduced the background which is caused by the reducing ends of soluble starch in the reaction mixture. The mixture of reduced soluble starch and reaction buffer were incubated at 40°C for 10 min initially and for a further 10 min after addition of 0.1 ml of enzyme solution. The enzyme solution was diluted to a concentration of 0.1 to 0.5 U/ml with 50 mM sodium acetate buffer, pH 5.7, containing bovine serum albumin at 0.4 mg/ml immediately before use in this reaction. Reducing sugar released by the enzyme was measured by the Somogyi-Nelson method.19 One unit (U) was defined as the amount of enzyme catalyzing the release of 1 μmol of maltose/min under these assay conditions.

Kinetic constants using the reduced soluble starch as substrate were calculated in the assay system mentioned above at substrate concentrations varying between 0.05 and 0.25% (w/v) by a Hanes-Wilkinson plot.20,21 The average molecular mass of the reduced soluble starch was measured by HPLC on a Shodex OHpak KB-80 M column using pullulan as a molecular-mass marker (Showa Denko Co., Japan). The non-reducing residues were measured by the method of Hizukuri et al.22 Thus, the substrate used here has the average molecular mass of 45,000 Da, so that one molecule contains 280 glucose residues, corresponding to 7.03 mol of non-reducing ends.

Protein was measured by the method of Bradford23 using a Bio-Rad protein assay kit. Crystalline bovine albumin (Seikagaku Kogyo Co., Japan) was used as a standard.

Mass spectrometric analysis. To measure the molecular masses of individual β-amylase isoforms, each fraction separated by chromatofocusing was put onto a reversed-phase HPLC column (Silica ODS 120T, Tosoh Co., Japan), equilibrated with 0.1% trifluoroacetic acid and eluted with a 60-min linear gradient of acetonitrile (0–60%) at a flow rate of 1 ml/min. The β-amylase protein was monitored by measuring the absorbance at 280 nm. The molecular masses of the desalted proteins were measured by using a Perkin-Elmer (PE) Sciex triple stage quadrupole ion spray mass spectrometer (model API 365) calibrated over the range of 200-3000 mass units (mu) using polypropylene glycols according to the manufacturer’s instructions.

Analyses of the N- and C-terminal amino acid sequences of the enzyme isoforms. The N-terminal sequencing of the pI-different isoforms was done using a PE-Applied Biosystems protein sequencer (model 477A) coupled with a PE-Applied Biosystems phenylthiohydantoin amino acid analyzer (model 120A) as described previously.24 The C-terminal sequencing runs were also done on a PE-Applied Biosystems Procise 494C sequencer by the method of Boyd, V. L. et al.25 An individual isoform protein (about 2 nmol) was put on a polyvinylidene difluoride (PVDF) membrane using a ProSorb Sample Preparation Cartridge and then automatically sequenced.
Results and Discussion

A further separation of authentic soybean β-amylase

Using this purification procedure, a recombinant β-
amylase was effectively purified to homogeneity as judged from analyses by SDS-PAGE (Fig. 1A, lane 3), isoelectric focusing (Fig. 1B, lane 3), the N- and the C-terminal amino acid sequencing (Table), and mass spectrometric pattern (data not shown). Thus, it is clear that the method was reliable for obtaining soybean β-amylase with a precisely purified state. According to this method, β-amylase was isolated from a partially defatted soybean flour. This enzyme preparation showed a single band corresponding to an apparent molecular mass of 56 kDa on SDS-PAGE (Fig. 1A), and the average yield was about 1.2 mg of enzyme protein from 20 g of the flour. For initial screening studies and analysis of a mixture containing the enzyme isoforms with differing isoelectric points, we chose an Ampholine PAGplate with a pH range of 3.5–9.5. However, we found that the authentic β-amylase preparation contains four isoforms, focusing into a very limited pH range such as 4.9–5.4, so that further separation of the isoform mixture was done in a preparative scale by chromatofocusing with the pH range 4–6.2. For pI measurement, Immobilin gel electrophoresis was used in a narrow pH range such as 4–7 (Fig. 1, lanes 2 and 7).

As shown in Fig. 2, three protein peaks were obtained from the enzyme preparation. For convenience, these fractions were designated as fractions 1–3 in the order of elution, and then the relative pI of each isoform was measured separately. Fraction 1 consisted of two isoforms with pIs of 5.32 (Isoform I) and 5.22 (Isoform II), and the amount was approximately 5% that of the authentic isoform mixture. Fraction 2 was composed of a single component having a pI of 5.09, named Isoform III, and the amount was about 76% that of the mixture, and fraction 3, containing only Isoform IV with a pI of 4.97, shared the remainder.

Characterization of pI-separated isoforms of soybean β-amylase

To analyze the physicochemical and enzymic characteristics of four β-amylase isoforms differing in pI, mass spectrometric analysis and N-terminal and C-terminal sequencing were done, together with the measurement of specific activity and kinetic parameters. Each mass spectrum obtained gave a single sharp reconstruction peak, indicating that highly similar protein, in terms of molecular mass, occurs in each of the three fractions. As shown in Fig. 3, Isoforms I and II had the identical molecular mass of 55,989 Da, and Isoform IV had 56,310 Da, while Isoform III, the largest quantity in the mixture of authentic β-amylase
Table. Comparison of Characteristics of Authentic β-Amylase Isoforms and pI-Separated Fractions 1-3 from the Authentic Enzyme Preparation

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Specific activity (U/mg)</th>
<th>( V_{\text{max}} ) μmol maltose/μg-min</th>
<th>( K_{\text{m}} ) mm</th>
<th>( k_{\text{cat}}/K_{\text{m}} ) s(^{-1}) mmol(^{-1})</th>
<th>pI</th>
<th>Molecular mass</th>
<th>N-terminal sequence</th>
<th>C-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic β-amylase</td>
<td>(1.2 ± 0.03) ( \times 10^5 )</td>
<td>(1.2 ± 0.02) ( \times 10^5 )</td>
<td>(7.7 ± 1.2) ( \times 10^{-2} )</td>
<td>(1.5 ± 0.26) ( \times 10^9 )</td>
<td>7.5</td>
<td>19,000</td>
<td>Val-Asp-Gly</td>
<td>Blocked</td>
</tr>
<tr>
<td>Fraction 1 (Isolform I and II)</td>
<td>(1.2 ± 0.02) ( \times 10^5 )</td>
<td>(1.2 ± 0.02) ( \times 10^5 )</td>
<td>(7.5 ± 1.0) ( \times 10^{-2} )</td>
<td>(1.5 ± 0.25) ( \times 10^9 )</td>
<td>5.32/5.22</td>
<td>55989</td>
<td>Blocked</td>
<td>Val-Asp-Gly</td>
</tr>
<tr>
<td>Fraction 2 (Isolform III)</td>
<td>(1.2 ± 0.02) ( \times 10^5 )</td>
<td>(1.2 ± 0.03) ( \times 10^5 )</td>
<td>(8.3 ± 1.0) ( \times 10^{-2} )</td>
<td>(1.4 ± 0.24) ( \times 10^9 )</td>
<td>5.69</td>
<td>55994</td>
<td>Blocked</td>
<td>Val-Asp-Gly</td>
</tr>
<tr>
<td>Fraction 3 (Isolform IV)</td>
<td>(1.2 ± 0.02) ( \times 10^5 )</td>
<td>(1.2 ± 0.03) ( \times 10^5 )</td>
<td>(8.1 ± 1.0) ( \times 10^{-2} )</td>
<td>(1.4 ± 0.23) ( \times 10^9 )</td>
<td>4.97</td>
<td>56310</td>
<td>Blocked</td>
<td>Val-Asp-Gly</td>
</tr>
<tr>
<td>Recombinant β-amylase</td>
<td>(0.3 ± 0.01) ( \times 10^5 )</td>
<td>(0.4 ± 0.01) ( \times 10^5 )</td>
<td>(15.2 ± 2.0) ( \times 10^{-2} )</td>
<td>(0.3 ± 0.04) ( \times 10^9 )</td>
<td>5.22</td>
<td>55927</td>
<td>Ala-Thr-Ser</td>
<td>Val-Asp-Gly</td>
</tr>
</tbody>
</table>

\( a \) Recombinant β-amylase, previously denoted as non-mutant II,\(^{16} \) was used as a reference.

\( b \) The specific activities are mean values ±SE for triplicate measurements in each of three individual experiments. The kinetic data such as \( V_{\text{max}} \), \( K_{\text{m}} \) and \( k_{\text{cat}}/K_{\text{m}} \) are mean values ±SE for duplicate measurements in each of three individual experiments.

isofoms, had a molecular mass of 55,994 Da, and the recombinant enzyme was 55,927 Da (Table). The molecular disparity of Isoforms I-III and the recombinant enzyme is explainable by the occurrence of an acetyl group at the N-terminus of the authentic β-amylase,\(^{26,27,28} \) because the recombinant is also processed to remove its initial Met during production in E. coli as well as the in vivo case.\(^{15} \) On the other hand, the molecular mass of Isoform IV was about 300 Da larger than those of any other isofoms found in the authentic enzyme preparation. This molecular disparity is beyond the error of mass spectrometric analysis (substantial deviation is less than 0.03% under these conditions.). Thus, soybean β-amylase isofoms may be classified into two groups, one of which includes Isoform IV having a larger molecular mass with a pI below 5.0 and another group that contains the remaining components (Isoforms I-III) having a molecular mass corresponding to the cDNA-deduced primary sequence with the acetyl group. The pI of individual isofoms belonging to the latter group is more than 5.0. The occurrence of molecular disparity in β-amylase isofoms may suggest the existence of a family of genes.

As presented in the Table, chemical analyses demonstrated that all of the four isofoms with differing pI have blocked N-termini and an identical C-terminal sequence, NH\(_2\)-Val-Asp-Gly-COOH, indicating that soybean authentic β-amylase does not undergo post-translational proteolysis except for releasing its initial Met. On the other hand, enzymic experiments for measuring specific activity and kinetic values showed that no significant difference occurs among these four isofoms. Under the conditions used here, experimental deviation (error) for specific activities was below 4% of the average values obtained from individual experiments, and the deviation for \( k_{\text{cat}}/K_{\text{m}} \) was below 18% of the average values. On the contrary, the specific activity of the recombinant enzyme decreased to a fourth of that for the authentic enzyme as well as a great decrease in the enzymic efficiency as reported previously.\(^{15} \)

Overall, soybean pI-differing β-amylase isofoms had an enzymic quality similar to one another, suggesting that there is no existence of soybean mRNA species encoding an enzyme isoform having lower functional efficiency. Further experiments are required to establish the full significance of our observations.

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References


