Isolation of Taka-amylose A Peptides Required for Substrate Binding

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An α-amylose from Aspergillus oryzae, Taka-amylose A (TAA), was cleaved into peptide fragments by an acid protease. Inactivation of TAA was greatly retarded by the addition of α-cyclodextrin or Ca²⁺. TAA peptide fragments were separated into two groups having no and high affinity to the substrate, soluble starch. This separation was done by the forced affinity chromatography method by a column of epichlorohydrin cross-linked soluble starch gel. Three peptides were isolated from the high-affinity fragments, purified by the ODS-120T column, and their amino acids were sequenced. Peptides I, II, and III originated from α-helix, β3-helix, and β-sheet, respectively, and all of these were located in the (β/α), barrel of the main domain of TAA molecule. A stereo graphic view showed that Peptides I–III were at the cleft near the catalytic site. Occurrence of a Trp residue in all three peptides strongly suggested that Trp was very important in the binding of TAA to the substrate, soluble starch.

**Key words:** α-amylose; forced affinity chromatography; substrate binding site, Taka-amylose A

A specific interaction of substrate and catalytic site of Taka-amylose A (TAA) has been described on the kinetic studies and X-ray crystallographic studies. Further insight into this aspect will provide useful information on the protein engineering of TAA to elevate the specific activity of TAA. In spite of the accumulation of knowledge on the raw starch binding site of Aspergillus niger glucoamylase and Pseudomonas stutzeri maltotetraose-forming exo α-amylase, we have no such information on TAA. However, it is interesting to note that, a competitive inhibitor of TAA, 6-deoxy-6-iodomaltotriose, bound at four different sites of the TAA molecule. Therefore, we attempted to isolate the substrate binding peptides from TAA.

An active site peptide was isolated from the glucosyltransferase of Streptococcus sobrinus. Dextran-binding peptides from the trypptic digests of dextran sucrose were also identified. In this paper, we describe the preparation of limited proteolized fragments of TAA by an acid protease, which contaminated in the purified TAA preparation. Substrate binding peptide was isolated with the forced affinity chromatography method. Based on the amino acid sequences of the isolated peptides, the substrate binding site of TAA was discussed.

**Materials and Methods**

Maltose Taka-amylose A (TAA) was the product of Sanko Co. Soluble starch and pepstatin and porcine pancreas α-amylase (PPA) were the products of Merck Co. and Sigma Co., respectively. Starch gel was prepared by cross-linking with epichlorohydrin as described. 

Assay of amylase and protease. TAA activity was measured as described. Acid protease activity was measured by the increase in the numbers of amino groups by using the fluorescent o-phthalaldehyde method.

**Acidic digestion of TAA.** TAA (40 mg/ml) dissolved in 20 mM acetic buffer, pH 5.2, was mixed with 0.2 M HCl-acetate buffer, pH 2.5-5.0 at the ratio of 1:9. After incubation at 30°C for suitable periods, a sample was removed and diluted 10-fold with 0.2 M acetic buffer, pH 5.2.

Effects of substrates and their analogs were measured with a reaction mixture containing 4% TAA (10 μl), substrate solution (100 μl), and 0.2 M acetate buffer, pH 3.0 (90 μl). The buffer solution was added after the incubation of TAA and substrate at 30°C for 5 min. The reaction was stopped by buffer dilution as described above.

Isolation of binding peptides from TAA. Protease digestion was done with a reaction mixture containing 4% TAA (100 μl), and 0.2 M HCl-acetate buffer, pH 3.0 (900 μl). After incubation at 30°C for 30 min, the reaction was stopped by the addition of 0.5 M sodium acetate solution. To this mixture, solid ammonium sulfate was added to the concentration of 2.3 M. This sample was placed in an affinity column of soluble-starch gel (1.3 x 5cm), which was equilibrated with 20 mM acetate buffer, pH 5.2, 2.3 M ammonium sulfate. The column was eluted with this buffer (1 ml fractions) and breakthrough fractions (P. 1) were collected by monitoring the absorbance at 280 nm. Then, the adsorbed fractions (P. 2) were eluted with 20 M acetate buffer, pH 5.2. Salts were removed by dialysis using a MW 3500 cut-off membrane (Micro-dialysis system, Bio-Tec Co.). Samples P. 1 and P. 2 were kept as the lyophilized powder.

Binding peptides were isolated with high pressure liquid chromatography (HPLC) apparatus (Jasco, PU-980) fixed a C 18 reversed-phase column (ODS-120T, Tosoh Co.), UV monitor (Jasco, 870-UV), and integrator (C-R6A, Shimadzu Co.). A gradient elution of 0.1% trifluoroacetic acid (TFA) to 0.1% TFA-70% acetonitrile with the flow rate of 0.8 ml/min was done. Elution profiles were monitored by UV at 220 nm.

**Amino acid sequence of peptides.** The lyophilized peptide fractions were dissolved in 30 mM NaCl-1 mM CaCl₂-50 mM phosphate buffer, pH 6.9 solution. To this mixture, 100 mM phenylmethylsulfonyl fluoride (PMSF) (5 μl) and α-amylase (PPA, 70 units/ml) (50 μl) were added and incubated at 30°C overnight. The reaction was stopped by 1 M NaOH (50 μl) and then the samples were further purified by re-chromatography with the ODS-120T column. Amino acid sequencing of the purified peptide samples was done with a Protein Sequencer 494 (Applied Bio-System Co.).

**Molecular modeling.** Graphical representation of the molecular structure of TAA were done on an Iris INDIGO R 4000 (Silicon Graphics Co., Ltd.) using the CHARMm (ver. 22) program (Molecular Simulations Inc.) established by Brooks et al.
Results and Discussion

Peptide isolation from TAA

TAA solution in 40 mM acetate buffer, pH 5.2, was highly stable and there was no significant loss of enzyme activity during the storage at 4°C over several months. However, a pH decrease in the TAA solution caused very rapid loss of activity (Fig. 1a). This inactivation was caused by the acid proteases such as aspartic proteinase and acid carboxypeptidase, which were tightly bound to the TAA preparation.91 Increase in the amino terminals was measured at pH 2.5–5.0 (Fig. 1b) and the optimum pH of acid proteases was observed at pH 3.0–3.5.

Inactivation of TAA was partially prevented by the addition of α-cyclodextrin (α-CD) as shown in Fig. 2. Other sugars tested including soluble starch, γ-CD, and maltose gave no significant protection from the protease action. Since α-CD had much smaller susceptibility to TAA than γ-CD142 and the proper size of numbers of glucosyl residues to fit the active site, good protection was given.

The effects of pepstatin, known as an inhibitor of acid proteases was examined. Although loss of TAA activity was not retarded, release of amino terminals was decreased to 57% with 2 μM pepstatin addition (data not shown). Moreover, addition of 25 mM CaCl₂ gave about 2/3 protection of TAA activity obtained by α-CD, where both α-CD and CaCl₂ gave almost the same level of retardation of protease activity. No effect was observed with KCl or EDTA in these measurements.
Identification of substrate binding site

Peptide fragments of TAA obtained by the action of acid proteases were separated into two groups having no (P. 1 fraction) and high affinity (P. 2 fraction) to the substrate soluble starch. Although in the absence of ammonium sulfate no binding of whole peptides occurred, substrate binding peptides were isolated by using a current forced affinity chromatography system. Peptide mapping of the P. 1 and P. 2 fractions was done by an HPLC column of ODS-120T. Comparison of the patterns of P. 1 and 2 with that of TAA peptides before the separation of the affinity column step showed the presence of at least three peptides, Peptides I–III, in the P. 2 fraction (Fig. 3). Since these particular peptides were recovered from the fraction bound to soluble starch gel, each peptide seemed to have high affinity for the substrate in spite of having no intact steric domain structure.

The amino acid sequence of the isolated Peptides I–III was determined (Table). Although Peptides I and III were too small to allow the assignment of a sequence, no other regions were found in the whole sequence of TAA. Before the rechromatography of Peptides I–III on the ODS-120T column, samples were treated with PPA (porcine α-amylase) to remove the small amounts of starch molecule, which possibly originated from the affinity gel matrix and severely interfered with the protein sequencer. A remarkable coincidence of the occurrence of a Trp residue at the amino terminals in all three peptide was noticed, suggesting that the Trp residues were important in the substrate binding of the TAA molecule.

All these peptides were located in the (β/α)8 barrel structure of the main domain of TAA. Peptides I, II, and III were on the 3rd α-helix, 4th α-helix, and 2nd β-sheet, respectively.

Roles of the isolated peptides in TAA

Proteolytic inactivation of TAA under the acidic pH condition was greatly restricted by the addition of α-CD (Fig. 2) or CaCl2. Binding of Ca2+ to TAA was mediated by the amino acid residues of Asn121, Asp175, and His-210, which were highly conserved in α-amylases from various sources. TAA contained 1 mol Ca2+/mol of enzyme.

<table>
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<th>Cycle</th>
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<th>Peptide II</th>
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* Position and A.A. corresponded to the sequence numbers and amino acids of peptides obtained for those of TAA.23

Fig. 4. Location of Peptides I–III in the TAA Molecule.
Peptide I, A; Peptide II, B; Peptide III, C; Catalytic residues: Asp 206, 1; Glu 230, 2; Asp 297, 3.
which bound at the cleft region of the active site and is known to protect the structure from damage such as heat denaturation.\textsuperscript{16} Substitution of His238 (His210 of TAA) to Asp caused a great decrease in heat stability of \( \varepsilon \)-amylase from \emph{Bacillus stearothermophilus}.\textsuperscript{17} Both Peptides I and II located close to the above Asp175 and His210 residues, respectively. Moreover, binding of 6'-deoxy-6'-iodo-maltotriose occurred at 4 Tyr residues at 113, 145, 155, and 188, and Cys227 in TAA.\textsuperscript{6} Peptide I contained the exact Tyr188 residue in the sequence and Peptide II led to the Cys227 residue by a short extrapolation to the carboxy terminal.

Location of the three isolated peptides is shown in Fig. 4. All peptides were very close to the active site, where the catalytic residues of Glu230, Asp206, and Asp297 were involved in close locations. Current results of the sequence analysis of substrate binding peptides provided definite evidence, which strongly supported the results obtained by the X-ray crystallographic analysis.\textsuperscript{23} TAA contained 9 Trp residues in the molecule. Interestingly all of Trp were in the main domain and mostly exposed to the surface of the molecule. Chemical modification with \( N \)-bromosuccinimide (NBS) showed that only one Trp residue (Trp83) was in the substrate binding site, probably at subsite 3.\textsuperscript{18}\textsuperscript{19} However, in this study, no Trp83 containing peptide was isolated (Table), possibly because of the very small size of the Trp83 peptide, which was formed by the acid protease action.\textsuperscript{19}\textsuperscript{20} Moreover, a significant increase in transglycosylation activity of \( \varepsilon \)-amylase from \emph{Saccharomyces fibuligera} was reported by the site-directed mutation of Trp84 to Leu(Trp83 in TAA).\textsuperscript{21} These results showed that all three peptides that have substrate binding ability, contained a Trp residue in the sequence, and the roles of these Trp residues in the catalytic mechanism might closely related to the above changes caused by the modification of Trp residue.

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\textbf{References}