Enhancement of the aspartame precursor synthetic activity of an organic solvent-stable protease

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The PST-01 protease is highly stable and catalyzes the synthesis of the aspartame precursor with high reaction yields in the presence of organic solvents. However, the synthesis rate using the PST-01 protease was slower than that observed when thermolysin was used. Structural comparison of both enzymes showed particular amino acid differences near the active center. These few residue differences in the PST-01 protease were mutated to match those amino acid types found in thermolysin. The mutated PST-01 proteases at the 114th residue from tyrosine to phenylalanine showed enhancement of synthetic activity. This activity was found to be similar to thermolysin. In addition, mutating the residue in the PST-01 protease with arginine and serine showed more improvement of the activity. The mutant PST-01 protease should be more useful than thermolysin for the synthesis of the aspartame precursor, because this enzyme has higher stability and activity in the presence of organic solvents. The results show the potential of organic solvent-stable enzymes as industrial catalysts.

Keywords: aspartame precursor/peptide synthesis/protease/reverse reaction/thermolysin

Introduction

Proteases mainly catalyze the hydrolysis of peptide bonds in aqueous solutions. However, the reaction is reversible and the equilibrium shifts toward the synthesis of peptide bonds in the presence of organic solvents. For example, aspartame, a low-calorific artificial sweetener, was enzymatically synthesized using a protease in the presence of organic solvents (Yagasaki and Hashimoto, 2008). Some proteases, including thermolysin, have been found to be capable of catalyzing the synthetic reaction of the organic solvent-stable enzyme, N-carbobenzyxoy-L-aspartyl-L-phenylalanine methyl ester (Cbz-Asp-Phe-OMe). Thermolysin has been well studied (Matthews, 1988). Although thermolysin is very stable at high temperatures, it is not very stable in the presence of organic solvents (Ogino et al., 1999a; Yagasaki and Hashimoto, 2008).

The PST-01 protease was found as an organic solvent-stable protease from the organic solvent-tolerant microorganism, Pseudomonas aeruginosa PST-01 (Ogino et al., 1995). In particular, the PST-01 protease is very stable in the presence of water-soluble organic solvents (Ogino et al., 1999a, 2007; Ogino and Ishikawa, 2001; Ogino, 2008; Doukyu and Ogino, 2010) and catalyzes peptide synthesis in the presence of organic solvents with both a high reaction rate and yield (Ogino et al., 1999b, 2000a; Bobe et al., 2004). Furthermore, the PST-01 protease has been shown to synthesize the aspartame precursor in the presence of organic solvents (Tsuchiyama et al., 2007).

In this work, the comparison of reaction rates of aspartame precursor synthesis using the PST-01 protease and thermolysin was performed. And enhancement of aspartame precursor synthetic activity of the PST-01 protease was attempted by generation of mutated PST-01 protease using site-directed mutagenesis. The mutation points were identified by structural comparison between the PST-01 protease and thermolysin. The synthesis rates of aspartame precursor using the mutated PST-01 proteases were measured and compared with wild-type PST-01 protease and thermolysin. Furthermore, site-saturated mutagenesis of the specific residue of the PST-01 protease was performed to construct mutated PST-01 protease which has high activity in the aspartame precursor synthesis and high stability in the presence of organic solvents.

Materials and methods

Construction of mutated PST-01 protease genes

Mutated PST-01 protease genes were constructed using a QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA, USA) and pPC1 containing the PST-01 protease gene (Ogino et al., 2000b) and its derivatives as templates. All mutated genes were confirmed by DNA sequencing.

Preparation of enzymes

Transformed Escherichia coli JM109 with pPC1 and its derivatives which harbored the wild-type and mutant PST-01 protease genes, respectively, were cultured in LB medium (pH 7.0) containing 50 mg/l ampicillin. A 500 ml baffled Erlenmeyer flask containing 200 ml of the medium was inoculated with the transformed cells and incubated at 37°C for 16 h with rotary shaking. The cells were collected by centrifugation and resuspended in a 10 mM Tris–HCl buffer (pH 8.0) and disrupted by intermittent ultrasonic disintegration at 97 W for 12 min in an ice bath. The supernatant was incubated at 30°C for 2 h and solid ammonium sulfate was added to 40% saturation. After removal of the precipitate formed by centrifugation, solid ammonium sulfate was further added to 75% saturation. The collected precipitate was dissolved in a 10 mM Tris–HCl buffer (pH 8.0) and
purified by hydrophobic interaction chromatography with a TSKgel Butyl-Toyopearl 650M column (Tosoh, Tokyo, Japan) as previously described (Ogino et al., 2000b). The homogeneity of the wild-type and mutated PST-01 proteases was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis. Thermolysin (protease type X from *Bacillus thermoproteolyticus* rokko, EC 3.4.24.27) was purchased from Sigma Chemical Company (St Louis, MO, USA). Concentrations of the purified proteases were determined by measuring the absorbances at 280 nm using bovine serum albumin as a standard.

**Peptide syntheses**

N-carbobenzoxy-L-aspartic acid (Cbz-Asp) from Kanto Chemical Co (Tokyo, Japan) and N-α-carbobenzoxy-L-arginine (Cbz-Arg) from Nacalai Tesque, Inc. (Kyoto, Japan) were used as carboxyl component substrates for the peptide syntheses. L-phenylalanine methyl ester (Phe-OMe) and L-leucinamide (Leu-NH₂) were used as an amine component substrates required for the peptide syntheses. Phe-OMe and Leu-NH₂ were prepared by the following method. A total of 30 mmol of L-phenylalanine methyl ester hydrochloride from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan) and 30 mmol of Na₂CO₃ were dissolved in distilled water (Ogino et al., 2000a). Phe-OMe and Leu-NH₂ were extracted with chloroform and were recovered by evaporating chloroform using a rotary evaporator at room temperature. Peptide syntheses were carried out using 1.0 ml of reaction mixtures containing 0.748–3.47 mg L-leucinamide (Cbz-Arg-Leu-NH₂), a mixture of acetonitrile and N,N-dimethyl sulfoxide (DMSO) at 30 °C. All reactions were carried out at 30 °C in a water bath.

**HPLC analysis**

The amount of synthesized products was analyzed by the following procedure: a small amount of sample solutions were taken from the reaction mixture after 0, 30, 60, 90, 120 and 180 min of incubation for the measurement of the reaction rates and at intervals of 24 h for the measurement of the equilibrium yields. Aliquots of sample solutions were diluted with an eluent [mixture of acetonitrile and 50 mM sodium phosphate buffer (pH 2.3)] for HPLC at a ratio of 1/20. The enzymatic reaction was terminated by the addition of the eluent and stored at −20 °C. An aliquot of the mixture was analyzed by reversed-phase HPLC. The column used was an ODS column packed with Cosmosil 5C18-AR-II (4.6 mm in diameter and 150 mm in height, Nacalai Tesque). To quantify the levels of Cbz-Asp-Phe-OMe and N-carbobenzyoxyl-l-aspartyl-l-leucinamide (Cbz-Asp-Leu-NH₂), a mixture of acetonitrile and 50 mM sodium phosphate buffer (pH 2.3) (33/67; v/v) was used as an eluent and a flow-rate of 0.8 ml/min. To quantify the levels of N-α-carbobenzoxy-L-arginyl-L-phenylalanine methyl ester (Cbz-Arg-Phe-OMe), a mixture of acetonitrile and 50 mM sodium phosphate buffer (pH 2.1) (23/77; v/v) was used as an eluent and a flow-rate of 1.0 ml/min. To quantify the production of N-α-carbobenzyoxyl-l-arginyl-l-leucinamide (Cbz-Arg-Leu-NH₂), a mixture of acetonitrile and 50 mM sodium phosphate buffer (pH 2.1) (20/80; v/v) was used as an eluent and a flow-rate of 1.0 ml/min. The oven temperature was 35 °C. The eluted reactants and product were detected at 254 nm. The product yield was calculated based on the amount of the limiting substrate, the carboxyl component. The initial rates were calculated from the slopes of the amount of product versus reaction time curves. When the concentrations of the carboxyl component and the product were the same as those measured 24 h earlier, the reaction mixture was assumed to have attained equilibrium.

**Results and discussion**

**Peptide synthesis using PST-01 protease and thermolysin**

High-yield synthesis of an aspartame precursor was attained when the PST-01 protease was used as a catalyst in the presence of 50% (v/v) DMSO in our previous paper (Tsuchiyama et al., 2007). However, the synthesis rate of the aspartame precursor using the PST-01 protease was about 16.5 ± 3.0 μmol·(min g-enzyme)⁻¹ and is approximately one-third of that using thermolysin [50.7 ± 1.1 μmol·(min g-enzyme)⁻¹] under the same reaction conditions. Product yields at equilibrium using either the PST-01 protease or thermolysin have been shown to be almost identical when the same reaction conditions were used.

**Structural comparison between PST-01 protease and thermolysin**

The major difference seemed to be the substrate specificity of both enzymes. The overall structure of the PST-01 protease is very similar to that of thermolysin (Fig. 1A and B) (Thayer et al., 1991; Holland et al., 1995; Ogino et al., 2000b, 2001). Comparison of the substrate-binding sites of the PST-01 protease and thermolysin was performed. The catalytic mechanism and substrate-binding site of the thermolysin have been investigated by pioneering research (Matthews, 1988). For thermolysin, Glu-143 in the HEXXH motif has been proposed as a key residue in the catalytic site (Weaver et al., 1977). Phe-114 and a hydrophobic pocket consisting of Phe-130, Leu-133, Val-139, Ile-188, Gly-189, Val-192 and Leu-202 have been proposed as the S₁ and S₁' subsites, respectively (Hangauer et al., 1984; Tiraboschi et al., 1999; de Kreij et al., 2001). Although the same motif of the catalytic center was conserved in the PST-01 protease, some residues that formed the substrate-binding site of the PST-01 protease were different from those of thermolysin (Fig. 1C and D). It was found that Phe-114 of thermolysin corresponds to Tyr-114 of the PST-01 protease and Phe-130, Leu-133, Val-139, Ile-188, Gly-189, Val-192 and Leu-202 of thermolysin correspond to Phe-129, Leu-132, Val-137, Ile-188, Gly-189, Val-192 and Leu-202 of the PST-01 protease. The catalytic mechanism and substrate-binding site of the PST-01 protease were different from those of thermolysin

**Mutated PST-01 protease having substrate-binding site similar to that of thermolysin**

By comparison of the active centers of the PST-01 protease and thermolysin, the differences were found to be located at...
Y114 and Ile-190 of the PST-01 protease. Tyr-114 and Ile-190 of the PST-01 protease correspond to Phe-114 and Val-192 in thermolysin, respectively. To examine the influence of these residues on enzymatic activity, Tyr-114 and Ile-190 of the PST-01 protease were replaced with Phe and Val, respectively. The genes of the mutated PST-01-Y114F and PST-01-I190V proteases, and the double-mutant PST-01-Y114F-I190V protease were generated by site-directed mutagenesis. The wild-type and mutated PST-01 protease genes were expressed and the expressed proteins were purified. The specific caseinolytic activity of the wild-type PST-01 protease was 38.1 ± 0.8 U/mg, whereas the activities of the PST-01-Y114F and PST-01-I190V proteases, and the double-mutant PST-01-Y114F-I190V protease were generated by site-directed mutagenesis. The wild-type and mutated PST-01 protease genes were expressed and the expressed proteins were purified. The specific caseinolytic activity of the wild-type PST-01 protease was 38.1 ± 0.8 U/mg, whereas the activities of the PST-01-Y114F, PST-01-I190V and PST-01-Y114F-I190V mutant proteases were 40.1 ± 0.4, 37.9 ± 0.2 and 39.8 ± 1.2 U/mg, respectively. As such, the effect of the mutations on the caseinolytic activity was negligible. For comparison, the specific caseinolytic activity of thermolysin was 20.2 ± 0.7 U/mg.

The peptide synthesis rates of the aspartame precursor were measured using the purified mutant PST-01 proteases. Although the catalytic activity of the PST-01-I190V mutant [15.3 ± 0.1 μmol-(min g-enzyme)^{-1}] was similar to the wild-type PST-01 protease [16.5 ± 3.0 μmol-(min g-enzyme)^{-1}], the activity of the PST-01-Y114F mutant [44.2 ± 0.1 μmol-(min g-enzyme)^{-1}] was greatly enhanced and similar to that of thermolysin [50.7 ± 1.1 μmol-(min g-enzyme)^{-1}]. The Y114F mutation increased the PST-01 protease synthetic activity by a factor of 2.7. The synthesis rate of the aspartame precursor of the double-mutated PST-01-Y114F-I190V protease [46.7 ± 7.3 μmol-(min g-enzyme)^{-1}] was similar to that using the PST-01-Y114F mutant protease and thermolysin.

**Site-saturated mutagenesis of residue 114 of PST-01 protease**

The mutations of the PST-01 protease showed the importance of residue 114 for the synthesis of the aspartame precursor. Site-saturated mutagenesis of residue 114 was subsequently performed. PST-01 protease mutants, in which the 114th residue was changed from Tyr to Asn, Asp, Gln, Glu, Gly, Lys and Pro, did not show catalytic activity. Consequently, mutation at position 114 of the PST-01 proteases to polar amino acids such as Asn, Asp, Gln, Glu, Gly, Lys and Pro, did not show catalytic activity. Consequently, mutation at position 114 of the PST-01 proteases to polar amino acids such as Asn, Asp, Gln, Glu, Gly, Lys and Pro, did not show catalytic activity. Consequently, mutation at position 114 of the PST-01 proteases to polar amino acids such as Asn, Asp, Gln, Glu, Gly, Lys and Pro, did not show catalytic activity. Consequently, mutation at position 114 of the PST-01 proteases to polar amino acids such as Asn, Asp, Gln, Glu, Gly, Lys and Pro, did not show catalytic activity. Consequently, mutation at position 114 of the PST-01 proteases to polar amino acids such as Asn, Asp, Gln, Glu, Gly, Lys and Pro, did not show catalytic activity.

**Fig. 1.** Structural comparison between the PST-01 protease and thermolysin. (A) Overall structure of the PST-01 protease. α-Helix, β-strand, active center and the disulfide bond are colored red, blue, green and yellow, respectively. The zinc and calcium ions are shown as brown and gray spheres, respectively. (B) Overall structure of thermolysin. Partial structure near the active site of (C) the PST-01 protease and (D) of thermolysin. The diagrams of the PST-01 protease and thermolysin were created using PDB code 1EZM (Pseudomonas elastase LasB) and 1LNF, respectively. *Pseudomonas* elastase LasB and PST-01 protease have identical amino acid sequences. Numbers from N-terminal of mature proteases were shown.
catalytic activity. Mutations at this position to Leu and Thr showed moderate catalytic activity. Here the activity was found to lie between the wild-type PST-01 protease and thermolysin. Mutations at position Tyr-114 to Met, His, Cys, Ala, Arg and Ser led to a significant increase in catalytic activity. In particular, the activities of the PST-01-Y114R and PST-01-Y114S mutant proteases were about 10 times higher than that of the wild-type PST-01 protease and about 3.4 times higher than that of thermolysin.

To measure the kinetics parameters of the synthesis of the aspartame precursor, Cbz-Asp-Phe-OMe, synthetic reaction rates of the PST-01 protease were measured using various concentrations of Cbz-Asp and an excess amount of Phe-OMe. The values of the $k_{\text{cat}}$ and $K_m$ are summarized in Table I. By mutating residue 114, the $k_{\text{cat}}$ value was greatly influenced and increased by ~10-fold. However, the effect of the mutation on $K_m$ values was small and an increase was measured. From this result, it appears that the mutation at position 114 did not influence the affinity between the enzyme and a substrate, but did affect the catalytic activity. The reason for this is unclear, but one possible hypothesis is that mutations may have affected conformation of active site.

**Peptide syntheses using various substrates**

Various dipeptide syntheses were performed using wild-type and mutated PST-01 proteases and thermolysin. Initial rates for the syntheses of Cbz-Asp-Phe-OMe (aspartame precursor), Cbz-Arg-Phe-OMe, Cbz-Asp-Leu-NH$_2$ and Cbz-Arg-Leu-NH$_2$ by various proteases are summarized in Fig. 3. The reaction rate of Cbz-Asp-Phe-OMe was greatly increased by mutating residue 114. However, except for the PST-01-Y114R protease, drastic changes in the reaction rates by the mutations were not observed in the peptide syntheses of Cbz-Arg-Phe-OMe, Cbz-Asp-Leu-NH$_2$ and Cbz-Arg-Leu-NH$_2$. The results of the four peptide syntheses showed that essentially Cbz-Asp-Leu-NH$_2$ and Cbz-Arg-Phe-OMe syntheses were the favored reactions for thermolysin and the PST-01 protease, respectively. By replacing Tyr-114 of the PST-01 protease with Arg and Ser, the activities increased to a similar level to that observed for thermolysin in Cbz-Asp-Leu-NH$_2$ synthesis. In the synthesis of Cbz-Arg-Leu-NH$_2$, only the Y114R mutation gave rise to an increase in the reaction rate. The mutations at position 114 of the PST-01 protease resulted in limited increases in the reaction rates, such as the PST-01-Y114R and PST-01-Y114S proteases in Cbz-Asp-Leu-NH$_2$ synthesis and the PST-01-Y114R protease in Cbz-Arg-Leu-NH$_2$ synthesis.

**Replacement of amino acid located adjacent to residue 114 of PST-01 protease**

Inouye et al. (1998) reported the need for aromatic residues at position 115 of thermolysin. Trp-115 in thermolysin is located adjacent to Phe-114 of the S$_1$ subsite and corresponds to Trp-115 in the PST-01 protease. Therefore, the influence of substituting the residue at position 115 was expected to affect the activity of the PST-01 protease. The PST-01-W115F and PST-01-W115Y mutant proteases were generated. These mutations did not influence the catalytic synthesis of the aspartame precursor (data not shown). The double-mutant PST-01 proteases, PST-01-Y114F-W115F and PST-01-Y114F-W115Y proteases were generated. These double-mutant PST-01 proteases showed similar synthetic activities as the PST-01-Y114F protease and thermolysin.

Thermolysin is a well-known thermostable protease. It has four calcium ions. The presence of the bound calcium ions contributes significantly to the high thermostability. However, the interaction between thermolysin and some calcium ions is weak and calcium ions are easily released from thermolysin. Although thermolysin is very stable in the presence of calcium ions, it is unstable in the absence of calcium ions. Therefore, the addition of excessive amounts of calcium ions to the reaction mixture of thermolysin is required to maintain catalytic activity (Feder et al., 1971; Voordouw and Roche, 1975; Dahlquist et al., 1976). Excessive amounts of calcium ions induce the generation of

![Fig. 2. Synthesis of Cbz-Asp-Phe-OMe using the various Y114 PST-01 mutants. The reaction rates and yields are represented by the gray and white histograms, respectively.](image)

<table>
<thead>
<tr>
<th>Protease</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (mM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PST-01 (wild type)</td>
<td>9.44</td>
<td>17.1</td>
<td>0.553</td>
</tr>
<tr>
<td>PST-01-Y114F</td>
<td>17.0</td>
<td>14.3</td>
<td>1.19</td>
</tr>
<tr>
<td>PST-01-Y114A</td>
<td>48.6</td>
<td>19.4</td>
<td>2.51</td>
</tr>
<tr>
<td>PST-01-Y114R</td>
<td>89.1</td>
<td>28.0</td>
<td>3.18</td>
</tr>
<tr>
<td>PST-01-Y114S</td>
<td>99.0</td>
<td>33.8</td>
<td>2.93</td>
</tr>
</tbody>
</table>
calcium scale which causes problems in industrial reaction processes, especially continuous chemical processes using a continuous stirred tank reactor and a plug flow reactor. This contrasts the PST-01 protease, while binding one calcium ion (Thayer et al., 1991; Ogino et al., 2000b) does not require the addition of excessive amounts of calcium ions for activity. The binding of the calcium ion on the PST-01 protease appears to be relatively strong. Therefore, industrial reaction processes of aspartame synthesis using the PST-01 protease have the advantage over using thermolysin due to the lower concentration of calcium ions required. The processes using the PST-01 protease would avoid potential calcium scale problems. The PST-01 protease is also more stable in the presence of organic solvents (Ogino et al., 1999a; Ogino and Ishikawa, 2001; Ogino, 2008) and as shown in this work, particular mutant PST-01 proteases showed higher aspartame precursor synthetic activities than thermolysin.

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