Note
Identification of N-terminal Autodigestion Target Site in Subtilisin ALP I

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Autodigestion of subtilisin ALP I (ALP I), secreted from the alkalophilic Bacillus sp. NKS-21 and its predicted amino acid sequence having about 60% identity with other alkaline subtilisins, was examined under alkaline conditions. At the alkaline pH of 12, ALP I was rapidly degraded, and no breakdown products were detectable. However, by incubating ALP I at 5°C for an extended time, a couple of specific peptides (26.7 kDa and 25.6 kDa) were accumulated. Each of them was purified and amino acid sequences of these fragments were found. Both peptides appeared to start at Gly-19 of the mature sequence of ALP I.

Keywords: serine protease; autodigestion; alkaline condition; alkaline stability

Subtilisins are serine proteases that originate from strains of Bacillus subtilis or related bacteria.1) We have previously isolated two types of subtilisins from alkalophilic Bacillus. A subtilisin Sendai (Sendai) from Bacillus sp. G825-69 showed extreme stability against alkaline condition at pH 12, on the other hand, a subtilisin ALP I (ALP I) from alkalophilic Bacillus sp. NKS-21 gradually lost its activity as pH increased to 11.0, accompanied by alkaline denaturation and protein degradation (Fig. 1).3,4)

To improve the alkaline stability of ALP I, replacement of amino acid residues of the major autolytic cleavage sites by site-directed mutagenesis is one possibility. Before this, it is necessary to identify the autodigestion target sites of ALP I.

In this note, we report the N-terminal amino acid sequence of the primary autolytic cleavage sites of ALP I.

Subtilisin ALP I was purified by the previously described method.3) Proteolytic activity was assayed by a fluorometric method using Suc-Ala-Ala-Pro-Phe-MCA as described previously.5) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done by the procedure of Laemmli.6)

Figure 2 shows the course of autodigestion of ALP I at pH 12.0 under normal (30°C, Fig. 2A) or lower incubation temperature on SDS-PAGE, stained with Coomassie Brilliant Blue R-250 (5°C, Fig. 2B). At 30°C, the amount of mature ALP I decreased rapidly upon incubation for 2 min and no breakdown products were detectable. On the other hand, at 5°C the mature ALP I was slowly degraded with successful accumulation of a specific breakdown peptide, which had an molecular mass of 26.7 kDa.

To prepare limited autolytic fragments, 500 μg of purified enzyme was dissolved with 2.5 ml of 5 mM phosphate buffer, pH 7.0. An equal volume of 200 mM borate-phosphate buffer, pH 12.0 was added to the enzyme solution (pH of the mixture was 12.0, according to preliminary experiments) and the mix-

Fig. 1. Effects of pH on Stability of Subtilisin ALP I and Subtilisin Sendai. Purified subtilisins were dissolved in 5 mM phosphate buffer, pH 7.0, to a final concentration of 0.2 mg/ml. An equal volume of 200 mM borate-phosphate buffer, pH 7.0, 10.0, 11.0, 11.2, 11.4, 11.6, 11.8, or 12.0, was added to the enzyme solution and the mixture was incubated at 30°C for 10 min. The relative activity at pH 10.0 with Suc-Ala-Ala-Pro-Phe-MCA were done at 30°C. The values are shown, compared with that at pH 7.0. Open circles and filled circles represent data for subtilisin ALP I and subtilisin Sendai, respectively.

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There was a left 5°C for 10 min. The reaction was stopped by adding equal amounts of 100% trichloroacetic acid, then the mixture was left on ice for 2 hr. The mixture was centrifuged 18,500 g at 4°C for 20 min, and the precipitates were dissolved with 100 μl of SDS-sampling buffer (0.12 M Tris-HCl buffer, pH 8.8 containing 5% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.05% BPB) and boiled for 5 min. The fragment mixture was put on a preparative SDS-PAGE column (15% polyacrylamide gel, 9 x 40 mm) and electrophoresed with constant current (5 mA), and the samples were collected at 3 min intervals. Figure 3 shows SDS-PAGE profiles of a one-thousandth part of each fraction, detected with silver staining.

Two specific peptides (peptide 1; 26.7 kDa and peptide 2; 25.6 kDa) in addition to the mature ALP 1 (28.8 kDa) were detected in the profile. The rest of the fractions containing each peptide were concentrated by trichloroacetic acid precipitation, and applied to SDS-PAGE and the peptide band appeared in SDS-PAGE was transferred to PVDF membrane using a semi-dry electrophoretic (Trans-Blot SD, BioRad). The Coomassie Brilliant Blue R-250 stained band was cut from the membrane and its N-terminal sequence was analyzed on Applied Biosystems 473A protein sequencer with 610A data analysis system.

The results of the N-terminal amino acid sequence analyses of two peptides are shown in Figure 4. Both peptides had the same N-terminal sequence of Gly-Tyr-Phe-Gly-Asn-Gly, indicating these peptides appeared to start at Gly-19 of the primary sequence of ALP 1. Identified fission sites of ALP 1 were in agreement with the known substrate specificity of the enzyme, showing a preference for the generation of fissions at the C-terminal side of large or hydrophobic residues, such as leucine, tyrosine, and glutamine. The estimated molecular mass (26.7 kDa) of peptide 1 suggests that its length is enough to contain the C-terminal region of mature ALP 1, while the smaller fragment (peptide 2; 25.6 kDa) lacks the C-terminal region of ALP 1. These results indicate that the initial fission site in ALP 1 under alkaline environment is located in the amino acid sequences between Gln-18 and Gly-19 of the mature enzyme.

Braxton and Wells have identified the site of autolysis in denatured subtilisin BPN' at elevated temperature, and they have identified two specific cleavage sites, Ala-48-Ser-49 and Ser-163-Thr-164. This suggests that the initial fission site in alkaline induced au-
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Fig. 5. Putative 3-D Structure Model of ALP I.
A three-dimensional ribbon model of ALP I was built exploiting the sequence homology with M-protease, 62.9% identity with ALP I as described previously. Model building were done using the program Swiss-Pdb Viewer. Wide ribbons indicate α-helix at N-terminal (Gly-6-Arg-17) and C-terminal (Glu-268-Gln-272), respectively. The side chains shown in bold line indicate Gln18 and Gly19, the initial autodigestion target site, at loop structure (Gln-18-Val-25) as shown as a black ribbon. Space-filled residues indicate the catalytic triad (Asp-31, His-61 and Ser-218) of ALP I.

Autodigestion of ALP I starts by elimination of the N-terminal α-helix fragment constituting 18 amino acid residues.

References