Enhancement of the Thermostability of the Alkaline Protease from *Aspergillus oryzae* by Introduction of a Disulfide Bond

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*Aspergillus oryzae* is used for manufacturing soy sauce and its alkaline serine protease (Alp) is considered to be important in producing the soy sauce. A full length cDNA for Alp has been cloned and the nucleotide sequence has been analyzed. The entire cDNA, coding for prepro Alp, when introduced into the yeast *Saccharomyces cerevisiae*, directed the secretion of enzymatically active Alp into the culture medium, with its N-terminus and specific activity identical to native *Aspergillus* Alp. One of the aims of protein engineering is to learn how to stabilize proteins to broaden their utility in medicine and commerce. An attempt to introduce a disulfide bond into a protein that enhances the stability of a protein was made by many courageous scientists. We report in this article that introduction of a disulfide bond by site-directed mutagenesis enhanced the thermostability of cysteine-free alkaline protease (Alp) from *Aspergillus oryzae*. Aqaulysin I is a thermostable subtilisin-type protease produced by *Thermus aquaticus* YT-1 and contains four Cys residues forming two disulfide bonds. The primary structure of Alp shows 44% homology to that of aqaulysin I. Sites for Cys substitutions to form a disulfide bond were chosen in the Alp based on the sequence alignment with aqaulysin I. In the case of the Cys-69/Cys-101 mutant Alp, Cys residues were introduced at positions 69 (wild type, Ser) and 101 (Gly) and the Cys-169/Cys-200 mutant Alp at positions 169 (Gly) and 200 (Val). Mutations were introduced into the Alp gene by site-directed mutagenesis (the Amersham system), using synthetic oligonucleotide primers having the sequence in Fig. 1: Wild-type and these mutants were expressed in *S. cerevisiae* and the enzymes secreted into the cultured medium were purified by the method of Tatsumi et al. Each of the Cys-69/Cys-101 and Cys-169/Cys-200 mutant Alps were found to form a disulfide bond by the conventional assay for sulphydryl groups in protein using 5,5'-dithiobis(2-nitrobenzoic acid). The replacement of Ser-69, Gly-101, Gly-169, and Val-200 by Cys was done with four oligonucleotide primers. The symbol * designates substituted nucleotide. The number designates the number of amino acids counted from the N-terminus of mature alkaline protease.

![Fig. 1](image1)

**Fig. 1.** The Sequence of Synthetic Oligonucleotide Primers.

![Fig. 2](image2)

**Fig. 2.** Course of Heat Inactivation at 50°C of Wild-type and Mutant Alps.

Enzyme solution was in 100 mM NaCl, 1 mM EDTA and 50 mM sodium acetate buffer (pH 6.0 at 25°C). Enzyme concentration was 20 μm. One ml of a 1% casein (Sigma) solution in 2 mM EDTA and 50 mM sodium phosphate buffer (pH 7.0) was mixed with 50 μl of an enzyme solution that had been incubated for time t (min) at 50°C and incubated at 30°C for 20 min, and 1 ml of 10% trichloroacetic acid were added to stop the reaction. The mixture was filtered and OD₄₅₀ of the filtrate was measured. The course of heat inactivation at 50°C is shown for (△) Cys-69/Cys-101 mutant Alp, (○) wild-type Alp, and (□) Cys-169/Cys-200 mutant Alp.

![Fig. 3](image3)

**Fig. 3.** The Effects of Temperature on the Proteolytic Activity of Wild-type and Mutant Alps.

The enzyme activity was measured at the indicated temperature for 10 min for 1% casein in 2 mM EDTA and 50 mM sodium phosphate buffer (pH 7.0). The assay method was described in legend to Fig. 2. The relative enzyme activity is shown for (△) Cys-69/Cys-101 mutant Alp, (○) wild-type Alp, and (□) Cys-169/Cys-200 mutant Alp.

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Some properties of the mutants were measured. Protease activity was measured by the method of Fukushima et al.11) One unit of protease activity (PU) was defined as the amount which catalyzed a release of 1 µg of tyrosine per minute from azo-casein.11) The specific activities of the purified yeast-Alp, Cys-69/Cys-101 mutant, and Cys-169/Cys-200 mutant were 490, 270, and 370 PU/mg of protein, respectively. Figure 2 shows the course of heat inactivation of Alp at 50°C. The semi-logarithmic plots of the heat inactivation of the enzyme gave a linear relationship with time. No dependence of the stability on concentration of the enzyme at pH 6.0 (data not shown) and the linear relationship shown in Fig. 2 led us the conclusion that the thermal inactivation of Alp was a first-order process and the autolysis did not take place in the range of enzyme concentration from 6 µM to 30 µM at pH 6.0. The half-life of heat inactivation at 50°C of wild type was 20 min and that of the Cys-69/Cys-101 mutant Alp was 13 min and that of the Cys-169/Cys-200 mutant Alp was 330 min, 13 times longer than that of wild type. This means that the introduction of a disulfide bond between positions 169 and 200 increased the thermostability of Alp. On the other hand the thermostability of the Cys-69/Cys-101 mutant Alp was decreased. Takagi et al.10) reported enhancement of the thermostability of subtilisin E (Cys-61/Cys-98 mutant) by introduction of a disulfide bond engineered on the basis of structural comparison with aqualysin I.

Figure 3 shows the effects of temperature on proteolytic activity of wild type and these Alps. The temperature at which the enzyme showed maximum activity was 51°C for wild Alp, 48°C for the Cys-69/Cys-101 mutant, and 56°C for the Cys-169/Cys-200 mutant Alp. The optimum temperature for activity of the Cys-169/Cys-200 mutant was raised from 51°C (wild type) to 56°C, indicating that the thermostolerance of the mutant Alp was increased.

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References