Study on thermostability of thermolysin

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Thermolysin (TLN, EC3.4.24.27), which is produced by *Bacillus thermoproteolyticus* and *B. stearothermophilus*, is a thermostable neutral protease binding of 4 calcium ions (Ca-1, -2, -3, -4) in its structure. Since TLN shows high stability, the enzyme is widely used for the industrial synthesis of an aspartame, and its stability and proteolytic mechanisms have been extensively investigated. Thermal inactivation of TLN at 90 ℃ was mainly caused through an autodegradation. In this study, autodegradation and calcium binding of TLN were investigated, and thermostable mutant TLNs were constructed.

Four autodegradation sites in TLN were identified in the presence of Ca^{2+}. One of the sites Leu^{155} was substituted with Ala, Ser, Phe, and Gly by site-directed mutagenesis. The thermostability at 80 ℃ increased in all mutant TLNs compare with WT TLN, and the autodegradation site shifted from the Gly^{154}-Leu^{155} to the Xaa^{155}-Ile^{156}. Furthermore, a new autodegradation site, Ile^{164}-Asp^{165}, was appeared in all mutant TLNs.

To construct further stabilized mutant TLN, the autodegradation resistant TLN was constructed by introducing mutations at the new autodegradation site of L155A. All mutant TLNs showed higher thermostability than L155A, and the autodegradation at Ala^{155}-Ile^{156} was suppressed.

Moreover, mutational effects for stability at the autodegradation sites of TLN in the conserved region were studied. Mutant TLNs in the conserved region only had the proteolytic activity in the culture supernatant in the case of cultivated in LB medium with 5 mM $CaCl_2$. Dialysis study shows that these mutant TLNs require more calcium-ions than WT TLN. Since the mutational sites were close to Ca-1, -2, and -4 binding sites, these conserved regions seem to play an important role for the binding of Ca^{2+}.

On the other hand, thermostable mutant TLN, I39F, was obtained by the random mutagenesis treatment. Although the mutational site was in the conserved region, mutant TLN became more stable without Ca^{2+} comparison with WT and L155A. These results indicate that enhancing calcium binding and changing amino acids around Ca-3 binding site have a possibility to construct more stable TLN.