Chapter 5

Engineering Thermolysin-like Proteases Whose Stability Is Largely Independent Of Calcium

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Summary

Thermal stability of the thermolysin-like protease produced by *Bacillus stearothermophilus* (TLP-ste) is highly dependent on calcium at concentrations in the mM range. We describe the rational design and production of a fully active TLP-ste variant whose stability is only slightly dependent on calcium concentration.

Introduction

Thermolysin-like proteases (TLPs) are a family of homologous metalloproteases that contain a catalytically important zinc ion in their active site. The three-dimensional structure of thermolysin is known (Holmes & Matthews, 1982) and this enzyme was shown to bind four calcium atoms which contribute to thermal stability (Dahlquist *et al.*, 1976; Roche & Voordouw, 1978). Two calcium ions are bound in the so-called double-calcium binding site (Ca1,2), that is composed of ligands that are conserved in all TLPs. The other, single binding sites (Ca3 and Ca4) are composed of ligands that are conserved only in the more stable TLPs such as thermolysin and the TLP produced by *B. stearothermophilus* (TLP-ste).

At elevated temperatures, TLPs are irreversibly inactivated as a consequence of autolysis. Autolysis follows first-order kinetics because its rate is determined by local unfolding processes that render the protease susceptible to autoproteolytic cleavage (Eijsink *et al.*, 1991b; Braxton & Wells, 1992; Vriend & Eijsink, 1993; Kidokoro *et al.*, 1995). In their studies on the contribution of calcium ions to thermolysin stability, Dahlquist *et al.* (1976) and Roche & Voordouw (1978) concluded that the initial steps in thermal inactivation are accompanied by the release of one calcium ion (Ca3 or Ca4). Extensive mutagenesis studies of the TLP-ste have shown that a region near the Ca3 site is crucial for thermal stability (Eijsink *et al.*, 1995; Veltman *et al.*, 1996). Thus, thermal inactivation seems to be dominated by one single 'weak' region, near Ca3. Considering the expected high structural similarity between thermolysin and TLP-ste (86 percent sequence identity) the studies on TLP-ste suggest that the critical calcium ion is Ca3 rather than Ca4.

Using the detailed knowledge about the thermal inactivation mechanism of TLPs that was gathered from a long series of mutation experiments, we set out for rational design of calcium-independent variants. The Ca3 site was deteriorated by mutating one of the main ligands (Asp57). Subsequently, the (expected) loss in stability was compensated for by introducing stabilising mutations in the direct environment of the Ca3 site. The results confirm the importance of the Ca3 site for stability and they show the feasibility of engineering less
calcium-dependent, stable TLP-ste variants.

Figure 1. Structure of the calcium binding site 3 (Ca3) in thermolysin (Holmes & Matthews, 1982). Crystal waters are indicated by crosses. Dashed lines indicate contacts of the Ca3 atom with surrounding residues and crystal waters. In the region depicted in this figure, the model is virtually identical to the crystal structure of thermolysin.

**Materials and methods**

*Production and characterisation of mutated enzymes*

Cloning, sequencing, sub-cloning, and expression of the TLP-ste gene, as well as production, purification and subsequent characterisation of wild-type and mutant TLP-ste were performed as described earlier (Takagi *et al*., 1985; Eijsink *et al*., 1992d). Thermal stability was measured as described previously (Eijsink *et al*., 1995; Veltman *et al*., 1996), using varying CaCl₂ concentrations in the standard assay buffer (20 mM Na acetate, pH 5.3, 0.01 % Triton X-100, 0.5 % isopropanol, 62.5 mM NaCl). T₅₀ is the temperature of incubation at which 50 percent of the initial proteolytic activity is lost during a 30 minutes incubation.

The kinetic parameter $k_{cat}/K_m$ (at 37 °C) for the substrate 3-(2-furylacryloyl)-L-glycyl-L-leucine-
amide (FaGLa, Sigma Chemical Company, St. Louis, MI., USA) was determined according to the method of Feder et al. (1969), in a buffer containing 50 mM Tris-HCl, pH 7.5, 5 mM CaCl$_2$, 5% DMSO, 1% isopropanol and 125 mM NaCl, using an 100 μM substrate concentration. Activities were derived from the decrease in absorption at 345 nm, using a $\Delta\varepsilon$ of -317 M$^{-1}$cm$^{-1}$.

**Structure analysis**

TLP-ste and thermolysin have 85% sequence identity which allowed the construction of a three-dimensional model of TLP-ste that is sufficiently reliable to predict the effects of site directed mutations (Vriend & Eijsink, 1993). The 55-69 region was expected to be highly similar in TLP-ste and thermolysin. Comparison of the two known TLP structures, thermolysin and the TLP from *B. cereus* (Stark et al., 1992) supported this: TLP-cer has lower homology to thermolysin (73% sequence identity) but, nevertheless has a strikingly similar fold in the 55-69 region (the RMSD positional difference is in the order of a few tenths of an Ångström, that is, in the order of the crystallographic error). Indeed, the TLP-ste model has been used successfully for the rational design of stabilising mutations (e.g. Hardy et al., 1993; Mansfeld et al., 1995). Structure analyses, three-dimensional modelling, prediction of the effects of point mutants, and data base searches were performed with the WHAT IF program (Vriend, 1990). Throughout this article residues are numbered according to the sequence of thermolysin.

**Results and discussion**

From a structural point of view Asp57 seemed more important for calcium binding than Asp59 because both O$\delta$s of Asp57 interact with the calcium versus only one O$\delta$ of Asp59 (Figure 1). Asp57 was replaced by Ser because in the less thermostable TLPs residue 57 is a serine. From a visual inspection of the three-dimensional environment of residue 57 it was concluded that the Asp57→Ser mutation would not have additional negative effects such as disturbance of the local hydrogen bonding network or the introduction of clashes. To compensate the expected destabilising effect of this mutation, the combined Thr63Phe-Ala69Pro mutation was chosen. This double mutation had previously been shown to drastically stabilise TLP-ste (Eijsink et al., 1995) and is located in the direct environment of Ca$^3$. Characteristics of the various mutants, including the dependence of stability on calcium concentration are presented in Tables 1 and 2 and in Figures 2 and 3. As shown in Table 2, the wild-type and mutant enzymes were similar with respect to their activity towards FaGLa.
Table 1. Thermal stabilities (T_{50}) at varying concentrations of CaCl_{2} of *B. stearothermophilus* thermolysin-like protease variants.

<table>
<thead>
<tr>
<th>[CaCl_{2}] (mM)</th>
<th>TLP-ste T_{50} (°C)</th>
<th>D57S T_{50} (°C)</th>
<th>T63F-A69P T_{50} (°C)</th>
<th>D57S-T63F-A69P T_{50} (°C)</th>
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<tr>
<td>0.2</td>
<td>65.5</td>
<td>65.9</td>
<td>77.8</td>
<td>74.6</td>
</tr>
<tr>
<td>0.5</td>
<td>67.5</td>
<td>67.3</td>
<td>80.9</td>
<td>75.4</td>
</tr>
<tr>
<td>2.5</td>
<td>72.9</td>
<td>67.5</td>
<td>68.6</td>
<td>76.2</td>
</tr>
<tr>
<td>5.0</td>
<td>75.2</td>
<td>68.2</td>
<td>87.2</td>
<td>76.5</td>
</tr>
<tr>
<td>7.5</td>
<td>77.0</td>
<td>68.8</td>
<td>89.7</td>
<td>76.7</td>
</tr>
<tr>
<td>12.5</td>
<td>77.9</td>
<td>69.4</td>
<td>90.2</td>
<td>77.2</td>
</tr>
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</table>

Error margins in the T_{50} values were in the range of 0.3-0.5 °C.

The Asp57→Ser mutation reduced the T_{50} of TLP-ste at 12.5 mM calcium from 77.9 °C to 69.4 °C (Table 1). In the stable Thr63Phe-Ala69Pro mutant the effect of the Asp57→Ser mutation was even more noticeable, and reduced T_{50} from 90.2 °C to 77.2 °C. Thus, the integrity of the Ca3 site is clearly important for TLP-ste's thermal stability.

Table 2. Activity and stability of TLP-ste variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>FaGLa, k_{cat}/K_{m} x10^{-3} (M^{-1}.S^{-1})</th>
<th>t/2 at 5 mM CaCl_{2} (min)</th>
<th>t/2 at 0.2 mM CaCl_{2} (min)</th>
<th>Half-life ratio (5 mM/0.2 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLP-ste</td>
<td>34</td>
<td>31</td>
<td>2.6</td>
<td>12</td>
</tr>
<tr>
<td>D57S</td>
<td>22</td>
<td>5.8</td>
<td>3.8</td>
<td>1.5</td>
</tr>
<tr>
<td>T63F-A69P</td>
<td>21</td>
<td>990</td>
<td>52</td>
<td>19</td>
</tr>
<tr>
<td>D57S-T63F-A69P</td>
<td>28</td>
<td>64</td>
<td>42</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The activity against FaGLa was determined at 37 °C. Half-lives (t/2) were calculated from the inactivation curves shown in Figure 3a and b. The t/2 value of the T63F-A69P mutation was extrapolated from the data presented in Figure 3a. The error margins in the t/2 values are less than 4 %.

The stability of TLP-ste and the Thr63Phe-Ala69Pro mutant (which both have the Ca3 site intact) depended strongly on the calcium concentration (Figure 2, Tables 1). Introduction of the Asp57→Ser mutation reduced this calcium dependence (Figure 2). Consequently, the
destabilising effect of the Asp57→ Ser mutant became smaller with decreasing calcium concentration (Table 2, Figure 3; at the lowest calcium concentration tested, the wild-type enzyme was even slightly stabilised by the Asp57→ Ser mutation). The stability versus calcium concentration curves of TLP-ste and Thr63Phe-Ala69Pro (Figure 2) can be superimposed remarkably well. The same is true for the Asp57→ Ser and the Asp57Ser-Thr63Phe-Ala69Pro (Figure 2), strongly suggesting that the observed effects on the calcium stability are indeed caused by the disturbance of the Ca3 site by the Asp57→ Ser mutation.

![Figure 2](image.png)

**Figure 2.** Effect of the calcium concentration on T50 for the wild-type (●), the D57S mutant (■), the T63F-A69P mutant (+) and the D57S-T63F-A69P mutant (▲). ΔT50 is the change in T50 upon lowering the calcium concentration. The T50 at 12.5 mM CaCl2 is used as reference value (ΔT50 = 0).

The fact that the Asp57→ Ser mutation largely abolishes the calcium-dependence of stability confirms earlier suggestions (Dahlquist et al., 1976; Roche & Voordouw, 1978; Eijsink et al., 1995) that calcium-3 is crucial in the process of thermal inactivation of TLPs. Apparently, the affinities of the other calcium sites are so high that titration effects are hardly noticeable at concentrations above 0.2 mM. We can not entirely exclude that the remaining effects of calcium on the stability of the mutants carrying Asp57→ Ser reflect titration of e.g. the Ca4 site. However, the dominant role of the Ca3 region in determining stability (e.g. Veltman et al., 1996; see also above) makes it more likely that the residual calcium-dependence reflects residual binding to the impaired Ca3 site (see Figure 1).
Figure 3. First order thermal inactivation of TLP-ste variants at 75 °C; in (A) 5 mM CaCl$_2$ and (B) 0.2 mM CaCl$_2$; wild type TLP-ste (●), D57S (■), T63F-A69P (+), D57S-T63F-A69P (▲).
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The Asp57Ser-Thr63Phe-Ala69Pro mutant represents a TLP-ste variant whose stability is largely independent on the calcium concentration and which, at lower calcium concentrations, is considerably more stable than the wild-type enzyme (Table 2). Combining known stabilising mutations in the Ca3 region has resulted in extremely stable TLP-ste variants (Eijsink et al., 1995). Therefore, it is likely that mutants can be designed that are even less dependent on calcium than the ones described here and that are more stable. Engineering calcium-independence does not necessarily need to be based on deteriorating the Ca3 site. Instead, it could be based on adding mutations that stabilise the local structure, regardless of the presence of a calcium ion. For example, preliminary analyses of a mutant in which the (intact) calcium binding site is covalently cross linked with the N-terminal $\beta$-hairpin (Mansfeld et al., 1995) showed that the stability of this mutant is also less calcium-dependent.

Results similar to those described here have been described by Strausberg et al. (1995) who engineered a calcium-independent, stable variant of the alkaline protease subtilisin BPN'. These authors first created a labile variant by deleting the high affinity calcium-binding site. Subsequently, semi-random mutagenesis and screening methods were used to isolate calcium-independent, stable variants. In the present study, detailed knowledge about the thermal inactivation of TLP-ste permitted the identification of the stability-determining calcium binding site and, subsequently, the fully rational design of a stable, calcium-independent mutant. Both studies show that the deleterious effect of impaired calcium-binding can be overcome by compensating mutations. This leads to the important conclusion that calcium-binding is a useful but certainly not unique way to obtain stable proteins.

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References

References are listed in chapter 9.