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Journal of Molecular Catalysis B: Enzymatic 22 (2003) 113–117
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Short communication

Improvement of thermostability of cold-active serine alkaline protease from the psychrotrophic bacterium *Shewanella* sp. strain Ac10 by rational mutagenesis

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Received 19 November 2002; received in revised form 10 January 2003; accepted 16 January 2003

Abstract

A serine alkaline protease (SapSh) from a psychrophilic bacterium *Shewanella* sp. strain Ac10 is a cold-active subtilase with low thermostability [Appl. Environ. Microbiol. 65 (1999) 611–617]. By means of homology modeling with other subtilase structures, we have constructed a mutant SapSh containing an extra salt bridge on its surface that exhibits higher thermostability and even higher $V_{max}/K_{m,app}$ value than those of the wild-type SapSh.

Keywords: Protease; Cold-active enzymes; Cold-adapted enzymes; Psychrophile; Thermostability; Homology modeling

Cold-adapted microorganisms produce cold-adapted enzymes that show specific activities much higher than those of their mesophilic and thermophilic counterparts at low temperatures such as 0–10 °C [1]. Due to their high catalytic efficiency at low temperatures, cold-adapted enzymes have numerous potential applications in the biotechnology industries [2]. However, the thermostability of these enzymes is generally lower than those of mesophilic and thermophilic counterparts, and this has precluded the establishment of their use as industrial enzymes. If the stability of cold-adapted enzymes could be enhanced without deteriorating their catalytic competence at low temperatures, these enzymes would prove useful in a wide variety of industrial applications. However, several site-directed mutagenesis studies suggested that the instability and "cold-activity" of cold-adapted enzymes may be inseparable from each other [3].

Recently, we isolated a gene coding for a serine alkaline protease (SapSh) from strain Ac10 of the genus *Shewanella*, a psychrotrophic bacterium [4]. We purified the recombinant enzyme to homogeneity from a culture filtrate of the transformant *Escherichia coli* cells and characterized it. It had properties typical of cold-adapted enzymes: high activity at low temperatures toward macromolecular substrates and low thermostability [4]. Primary structure analysis showed that SapSh should be a member of the superfamily of subtilisin-like serine proteases (subtilase [5]; EC 3.4.21.) with an overall sequence similarity of 25–28%

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doi:10.1016/S1381-1177(03)00012-2

to the other members of the family. In this study, we have attempted to engineer, by rational approaches, a SapSh mutant with enhanced thermostability and enhanced catalytic efficiency at low temperatures.

A mature form of SapSh (426 amino acid residues in length) has the putative catalytic domain exhibiting appreciable sequence similarities (40–48% identity) to subtilases with different thermostabilities, such as subtilisin BPN′ [6], subtilisin Carlsberg [7], and thermitase [8] (Fig. 1). Thus, as the first step to engineer more thermostable SapSh, a three-dimensional (3D) structural model for the putative "catalytic core" of SapSh, which lacks the 152-residue insert [4] (see also Fig. 1), was constructed on the basis of the crystal structure of subtilisin BPN′ [6] and compared with those of these subtilases (Fig. 1). Homology modeling was performed by means of a program MODELER version 4 (Accelrys, San Diego, CA, USA) that was run on a Silicon Graphics Indigo2 workstation. The model was generated by complete optimization cycles by means of conjugate gradients and simulated annealing. The quality of the structure was examined by PROCHECK (Accelrys). The final model was evaluated with Protein Health and 3D profile modules of QUANTA version 4.0 (Accelrys). All other estimations of structural parameters were done with software packages QUANTA version 4.0 or Insight II (Accelrys). A salt bridge was defined as an ion pair with a distance of 2.5–4 Å between charged non-hydrogen atoms [9]. A distance cutoff was applied to carboxylate oxygen atoms of Glu and Asp; NE, NH1, and NH2 of Arg; NZ of Lys; and ND1 and ND2 of His. The rotamer conformations of charged residue pairs were taken from QUANTA rotameric libraries and checked for the possibility of salt bridge formation.

We have predicted that the thermostabilities of subtilases are strongly correlated with the number of salt bridges: thermitase, subtilisin Carlsberg, and subtilisin BPN′ contain 10, 3, and 5 salt bridges, respectively, whereas the 3D model of SapSh has only one salt bridge. These observations in turn have raised the possibility that the introduction of additional salt bridge(s) may enhance the thermostability of SapSh in the same manner as previous reports [10]. Most importantly, we presumed that introduction of a salt bridge at a site far from the active site may minimally affect the cold-activity [11–13], resulting in a more stable SapSh with its characteristic cold-activity. A close inspection of the 3D model predicted that introduction of the Asn251*Glu mutation into SapSh (Fig. 1) should restore a salt bridge Arg247*Glu251* (originally present in subtilisin BPN′ and thermitase, see also Fig. 2) and may have such effects.

The Asn251*Glu substitution was introduced into the plasmid pSapSh3 encoding the full-length SapSh gene by PCR according to the overlap extension method of Ito et al. [14]. Using DNA sequencing, we verified that the mutations were introduced only into desired positions. The wild-type and the Asn251*Glu mutant SapShs were assayed with azocasein as a substrate, expressed in *E. coli* cells, and purified to homogeneity in the same manner as described previously [4]. The molecular mass of the purified SapSh was determined to be 43,709 Da by ion-spray mass spectrometry with a PE-Sciex API III mass spectrometer (Sciex, Thornhill, Ont., Canada). This value exactly corresponds to the 426 amino acid SapSh sequence extending from the N-terminal Ala1* to the C-terminal Ala275* [4]. The Asn251*Glu mutant showed a mobility which was indistinguishable from that of the wild-type SapSh by SDS-PAGE analysis [15], suggesting that the mutant should also undergo processing in the same manner as the wild-type enzyme.

To analyze the effect of the Asn251*Glu mutation on the thermostability of SapSh, the wild-type and mutant enzymes were incubated in 50 mM Tris–HCl (pH 8.5) containing 2 mM $CaCl_2$ at 55 °C. At appropriate time intervals, aliquots were withdrawn and placed into tubes in ice and assayed for remaining activity. The courses of inactivation of the wild-type and mutant SapShs followed pseudo-first order kinetics with an apparent half-life values of 38 ± 2 and 59 ± 4 min, respectively, indicating that the thermostability of the Asn251*Glu mutant was higher than that of the wild-type enzyme. Thus, the thermostability of SapSh could be enhanced by introduction of an intramolecular salt bridge that is located far from an active site.

A steady-state kinetic analysis of the reactions catalyzed by the wild-type and mutant SapShs was performed over a temperature range of 4–30 °C. The results showed that the V_{max} values of the Asn251*Glu mutant decreased by 32–56% compared with those of the wild-type enzyme (Table 1). Importantly, however, the catalytic efficiencies ($V_{max}/K_{m,\,app}$) of the

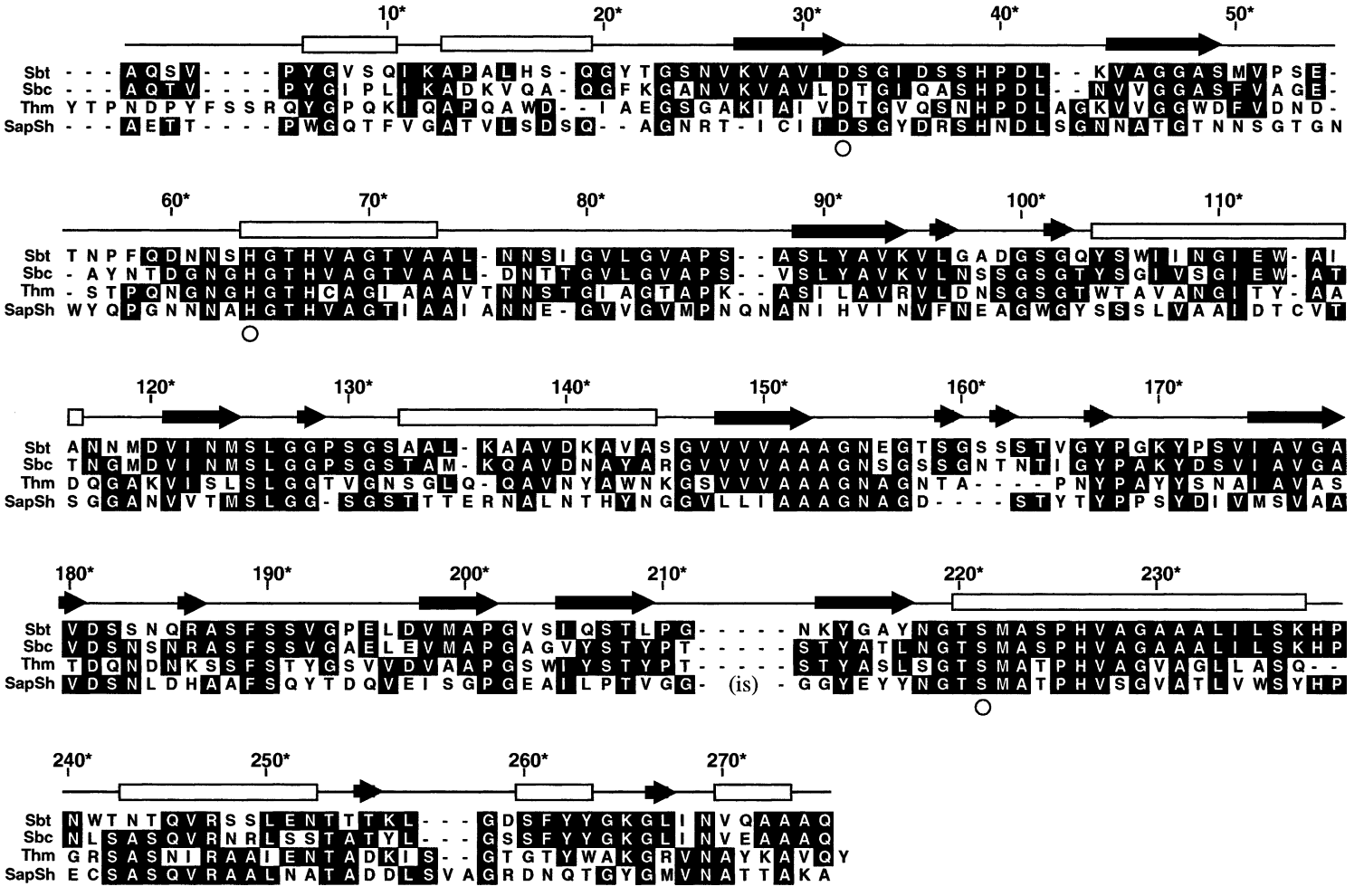

Fig. 1. Amino acid sequence alignment of the catalytic domains of subtilisin BPN′ (Sbt), subtilisin Carlsberg (Sbc), thermitase (Thm), and serine alkaline protease from *Shewanella* Ac10 (SapSh). Identical residues are shown in white-type on a black background. Amino acid residues are numbered so as to correspond to the numbering of subtilisin BPN′ on the basis of this alignment, and positional numbering, according to this notation, is indicated by an asterisk. The catalytic residues, Asp32*, His64*, and Ser221*, are indicated by open circles under the sequences. The location of the unique 152-residue insertion in SapSh is indicated with "is" in the SapSh sequence, which was omitted in the modeling studies. Secondary structures (β-strands and α-helices) of subtilisin BPN′ are denoted by arrows and rectangles, respectively, above the Sbt sequence.

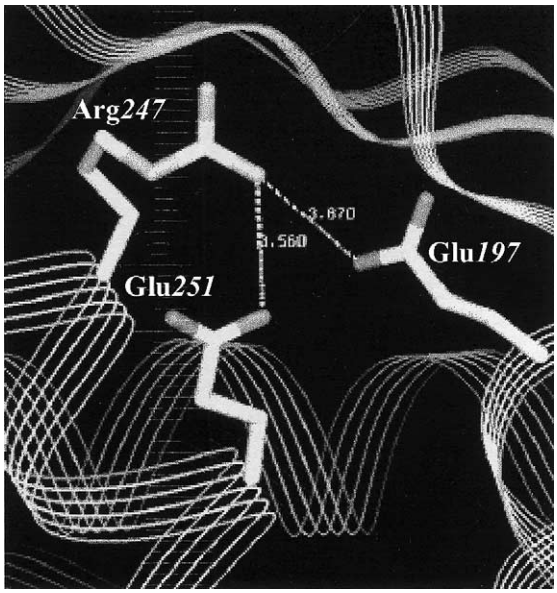

Fig. 2. Close up view of Asn251*Glu mutant. The mutated residues and those engaged in salt bridge formation are shown by sticks.

Asn251*Glu mutant were 1.3–3-fold higher than those of the wild-type SapSh in the temperature range of 4–30 °C. The $V_{max}/K_{m,app}$ value represents the first order rate constant at very low substrate concentrations ($\ll K_{m,app}$) and this observation could be explained in terms of a retained (or even enhanced) local flexibility of the active site for cold-activity under such conditions. These results provide good evidence for the possibility to create enzymes with enhanced thermostability and unchanged (or even improved) cold-activity.

The wild-type enzyme underwent substrate inhibition at high substrate concentrations ($\gg K_{m,app}$), which could be explained in terms of the non-productive binding of a second substrate molecule to the "enzyme–substrate" complex, as have also been observed with other subtilases [17,18]. It was observed that the Asn251*Glu mutation caused the changes in affinity of the "enzyme–substrate" complex for non-productive binding of substrate (see Table 1), suggesting that this mutation should cause an alteration of the conformation of the "enzyme–substrate" complex, and this may be related to the small diminution of V_{max} value upon mutation. These results may illustrate the complex nature of structural changes induced upon mutation: even if the substitutions occur far from an active site, they may affect the stability, catalytic action, and protein–protein interactions of an enzyme, probably through long-range interactions that result in changes in the mobility of different areas of an enzyme. Although the possibility of such unpredictable effects upon mutation must be considered, the present results prove the validity of our rational approach—rigidifying the enzyme by introducing salt

Table 1
Kinetic parameters of wild-type SapSh and the Asn251*Glu mutant[a]

	K_m (mg ml^{-1})	V_{max} (chromophore production, s^{-1})	V_{max}/K_m (ml s^{-1} mg^{-1})	K_i (mg ml^{-1})
Wild-type (°C)				
4	0.21 ± 0.05	1.7 ± 0.3	8.6 ± 0.9	13.4 ± 3.1
20	0.29 ± 0.07	5.8 ± 1.3	20.8 ± 1.6	4.6 ± 0.5
30	0.46 ± 0.09	10.5 ± 0.6	24.8 ± 4.4	ND[b]
Asn251*Glu (°C)				
4	0.049 ± 0.009	1.1 ± 0.2	23.1 ± 2.5	16.2 ± 0.7
20	0.15 ± 0.04	3.4 ± 0.3	25.5 ± 3.6	ND[b]
30	0.10 ± 0.03	7.1 ± 0.4	81.5 ± 24.3	ND[b]

[a] A steady-state kinetic analysis was performed with azocasein as a substrate. The amount of chromogen liberated from azocasein was determined by measuring absorbance at 366 nm [4]. An extinction coefficient of the chromophore, $\varepsilon_{366} = 900\ M^{-1}\ cm^{-1}$, was used for calculations. Hyperbolic or non-hyperbolic kinetics was obtained, depending on the assay temperatures and enzymes. The non-hyperbolic kinetics, where the specific activity decreased at high substrate concentrations, could be consistently explained in terms of substrate inhibition [16], where the binding of a second azocasein molecule to the enzyme at saturating substrate concentrations yields a non-productive complex [17,18]. The $K_{m,app}$ value, the inhibition constant, K_i, and the V_{max} value were determined as described previously [16]. The non-linear least square fit of the data was performed using Kaleida Graph (Synergy Software, Reading, PA). Values are means ± standard errors of three–four independent experiments.

[b] ND, the inhibition was not detectable in the investigated substrate concentration range 0.075–7.5 mg ml^{-1}.

bridge(s) at a local site far from the active site—as one possible strategy for engineering cold-active enzymes with enhanced thermostability, and this approach would enhance the usefulness of cold-active enzymes in biotechnology.

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