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Enhanced activity and stability in the presence of organic solvents by increased active site polarity and stabilization of a surface loop in a metalloprotease

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***Salinivibrio* zinc-metalloprotease (SVP) is an enzyme which was isolated from *Salinivibrio proteolyticus*, a moderately halophilic species from a hypersaline lake in Iran. A195E and G203D mutants were constructed to increase polarity near the active site in order to preserve the hydration layer against organic solvents [dimethylformamide (DMF), methanol, isopropanol and *n*-propanol]. A268P was constructed to stabilize a surface loop far from the active site and A195E/A268P was constructed to investigate the combined effects of these two mutations. Results showed that relative C_{50} values of A195E increased to ~26 and 11% in DMF and methanol whereas an increase of ~32 and 41% was observed in the presence of isopropanol and *n*-propanol. The irreversible thermoinactivation rate (k_i) for A195E was estimated to be 60 and 130 ($\times 10^{-3}\,min^{-1}$) in the presence of DMF and *n*-propanol, respectively, while k_i for SVP was 90 and 190 ($\times 10^{-3}\,min^{-1}$). G203D exhibited similar k_i as A195E in the presence of methanol and isopropanol, but the calculated k_i in the presence of DMF and *n*-propanol was 70 and 160 ($\times 10^{-3}\,min^{-1}$), respectively. A268P and A268P/A195E variants marginally increased the thermoresistance of the enzyme in this condition.**

Keywords: activity and stability/organic solvent/site directed mutagenesis/zinc-metalloprotease.

Abbreviations: FAGLA, *N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide; IPTG, Isopropyl-ß-D-1-thiogalactopyranoside; PWO, DNA Polymerases from *Pyrococcus woesei*; SVP, *Salinivibrio* zinc-metalloprotease; TLPs, Thermolysin-like proteases.

Enzymes have been successfully employed to catalyse a number of transformations and chiral resolutions of biological and industrial importance in organic solvents (*1*–*6*). These approaches require enzymes that are stable in the presence of organic media, but these solvents often drastically reduce both the stability and activity of enzymes (*7*). Several methods such as medium and/or protein engineering have been employed to improve enzymes for synthesis in organic media (*8*). However, a few reports are available in the literature concerning the site-directed mutagenesis of the enzyme in the presence of organic solvents. To obtain efficient biocatalysts which work in the presence of organic media with high activity and/or stability, random mutagenesis via directed evolution were attempted by several investigators (*9*–*16*). They showed that effective mutations were located at the variable surface loops and were all clustered in a region that encompasses the active site and substrate-binding pocket. This information can be used to develop more rational strategies for mutagenesis.

Proteases are one of the industrially most important enzymes and account for nearly 60% of total worldwide enzyme market (*17*). Hydrolytic enzymes such as proteases can also catalyse synthetic reactions, which are the reverse reactions of hydrolysis, in the presence of organic solvents (*18*). *Salinivibrio* zinc-metalloprotease (SVP) belongs to thermolysin (TLN)-like proteases (TLPs) or neutral metalloproteases. All members of this family contain the N-terminal domain enriched β-sheet and C-terminal domain enriched α-helix and active site formed at the junction of the two domains (*19*). To date, this zinc-metalloprotease (SVP) is the first amino acid sequences of proteases from moderately halophilic bacteria. SVP is active at alkaline pH (optimum 8.5–10) and over a wide range of salt concentrations (0–4 M) (*20*). Tolerance to high salt concentrations as well as stability at alkaline pH might be of particular importance for industrial applications (*21*). Therefore, improving the activity and stability of this enzyme in organic solvents is valuable for synthetic industrial biotechnology. The aim of this work was to study the effect of introducing surface charged residue and stabilizing surface loop near the active site on the enzyme activity and stability in organic solvents.

Experimental procedures

All experiments were performed at least in triplicate and the results were presented as their mean value. The experimental error was never over 7%.

Material

Oligonucleotides were synthesized by MWG (Germany). PWO (DNA polymerases from *Pyrococcus woesei*) was purchased from Roche

(Germany). *Dpn*1, IPTG (Isopropyl-ß-D-1-thiogalactopyranoside) and PCR reagents were supplied by Fermentase (Germany). FAGLA (*N*-[3-(2-furyl)acryloyl]-glycyl-L-leucine amide) was purchased from Sigma (USA). Q-Sepharose and Sephacryl S-200 were obtained from Amersham Biosciences. Organic solvents [dimethylformamide (DMF), methanol, isopropanol and *n*-propanol) were of analytical grade.

Bacterial strains, plasmid construction and transformation

SVP gene was previously subcloned into pQE-80L, an expression plasmid (*20*). *Escherichia coli* XL1 Blue and *E. coli* BL21 (DE3) were used as hosts for cloning and protein expression, respectively.

Site-directed mutagenesis

Site-directed mutagenesis on SVP gene was performed as described by Fisher and Pei (*22*). Three single mutants (A195E, G203D and A268P) and one double mutant (A195E/A268P) were introduced. The nucleotide sequences were confirmed by DNA Sequencer Company (MWG, Germany).

Expression of SVP protease and its mutants and their purification

Escherichia coli BL21 cells were transformed with pQ80L and used as a host for the production of wild-type and mutants SVP protease. *Escherichia coli* BL21 recombinant cells were cultured at 37°C and 224 rpm in 250 ml LB medium containing ampicillin (100 mg ml^{-1}) until an optical density of 0.5 at 600 nm was reached. Subsequently, IPTG was added to a final concentration of 1 mM, and culture was further incubated at 30°C with 250 rpm. After 24 h of incubation, cells were harvested by centrifugation (10,000*g*, 10 min). The supernatant was concentrated using a 10 kDa cut off amicon filter (Millipore) and dialysed against 20 mM Tris/HCl buffer, pH 8.5, overnight. The dialysed solution was purified by Q-Sepharose fast Flow column (Pharmacia) with a linear gradient of 0–0.5 M NaCl in 20 mM Tris/HCl buffer, pH 8.5. Active fractions were eluted at 0.15 M NaCl in 20 mM Tris/HCl buffer, pH 8.5, and the purity of the protein was confirmed using SDS–PAGE (12.5% acrylamide) as described by Laemmli (*23*). Active fractions from this step were concentrated and loaded on a Sephacryl S-200 gel filtration column, pre-equilibrated with the same buffer containing 0.15 M NaCl. Fractions with protease activity were pooled and concentrated. Protein concentration was determined by the Bradford method (*24*).

Protease assay in aqueous and organic solvent media

Protease activity was determined using FAGLA, as a synthetic substrate of TLPs (*25*, *26*). Hydrolysis of FAGLA by SVP and mutants was measured following the decrease in absorbance at 345 nm (*27*, *28*). The amount of FAGLA hydrolyzed was evaluated using the molar absorption difference due to hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\,cm^{-1}$, at 25°C (*27–29*). The reaction mixture (400 µl) contained 50 mM Tris, pH 7.0, 5 mM $CaCl_2$, 0.01% Triton X-100 and 50 µl of 20 mM FAGLA, dissolved in dimethylsulphoxide. The final concentration of the enzyme was 20 µg ml^{-1}. For determining protease activity in the presence of organic solvent, water/organic solvent mixtures were prepared by mixing required amounts of the components (organic solvents, aqueous buffer and substrate) and the enzyme (*30*). After preparing different concentrations of organic solvent the pH value was controlled and adjusted to the required value. The effect of increasing organic solvent up to 40% (V/V) on the enzyme activity was investigated under standard assay condition as described earlier. Activity is expressed as the remaining protease activity relative to control without any organic solvent (100%). C_{50} is the value of solvent concentration where 50% of enzyme activity remains and relative C_{50} values were determined from this equation [(C_{50} value of mutant $-C_{50}$ value of SVP)/C_{50} value of SVP] × 100. Kinetic constants for wild-type and mutant SVP were determined from a series of initial rates at different concentrations of FAGLA (0.02–2.5 mM). One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol min^{-1} product. All measurements were performed in at least three independent experiments. The standard deviations from the averages which are shown in the figures were ≤7%.

Thermal inactivation of SVP and mutants in organic solvent media

Purified proteases in 50 mM Tris–HCl, pH 7, containing 40% (V/V) of organic solvent were incubated at 60°C for different times (*31*). At various time intervals (2, 5, 10, 15, 20 min), samples were removed and cooled on ice. Subsequently, the residual activities were determined as described previously. In assay solution, the final concentrations of organic solvent and enzyme were 4% (V/V) and 20 µg ml^{-1}, respectively. The activity of the enzyme/organic solvent mixture that kept on ice was considered as control (100%). Plots of the log of residual protease activity versus time were linear, indicating a first-order decay process under these conditions. So, the rate of irreversible thermoinactivation (k_i) of SVP and mutants were measured.

Organic solvent stability of the SVP and mutants

The protease stability in the presence of organic solvent was examined by incubating filter-sterilized purified enzyme with organic solvent at 30°C, 160 rpm for 15 days (*31*, *32*). The final concentration of organic solvent in the incubation mixture was 40% (V/V). After the addition of the solvent, at appropriate time intervals (1, 2, 3, 4, 5, 7, 10, 12, 15 days), 50 µl from each sample were used for residual protease activity as described earlier. The final concentrations of organic solvent and enzyme in the assay mixture were 4% (V/V) and 20 µg ml^{-1}, respectively.

Circular dichroism studies

Circular dichroism (CD) experiments were carried out on a Aviv model 215 CD spectrophotometer using 20 mM Tris, pH 7. Far-UV CD (200–250 nm) was monitored using a cell of 2 mm path length with an enzyme concentration of 0.25 mg ml^{-1}. Results are expressed as molar ellipticity [θ] (deg cm^2 dmol^{-1}) based on a mean amino acid residue weight (MWR) assuming average weights of 110. The molar ellipticity [θ] was calculated from the formula $[\theta]_\lambda = (\theta \times 100\text{MWR})/(cl)$, where c is the protein concentration in mg ml^{-1}, l is the light path length in centimetres and θ is the measured ellipticity in degrees wavelength λ.

Fluorescence studies

Fluorescence measurements were carried out using a Cary Eclipse Spectrophotometer with an enzyme concentration of 20 µg ml^{-1} in 20 mM Tris buffer, pH 7. The sample was allowed to equilibrate for 5 min. The excitation wavelength was 280 nm and the emission spectra were recorded from 300 to 400 nm. The excitation and emission slits were set to 5 nm.

Results and Discussion

A three-dimensional model of SVP was built with Modeller, using the crystal structure of elastase from *Pseudomonas aeruginosa* (PAE) as a template (*33*). The 61% sequence identity between SVP and PAE indicates that SVP model is sufficiently reliable for prediction and analysis of the effect of most amino acids substitutions. Substitutions were designed as follows: (i) in order to increase water bonding sites and active site polarity, and to preserve the hydration layer of the enzyme during catalysis, A195E and G203D variants were constructed. These residues are located on the surface loops and close to the active site of SVP. (ii) A268P was designed to stabilize a surface loop adjacent to the substrate-binding pocket. (iii) Double mutant (A195E/A268P) was also constructed to consider the effect of the both mentioned criteria at the same time (Fig. 1). The nucleotide sequence of mutated plasmids were confirmed and transformed to *E. coli* BL21, inoculation with 1 mM IPTG and finally cells were harvested by centrifugation. The concentrated supernatant was dialysed and purified by

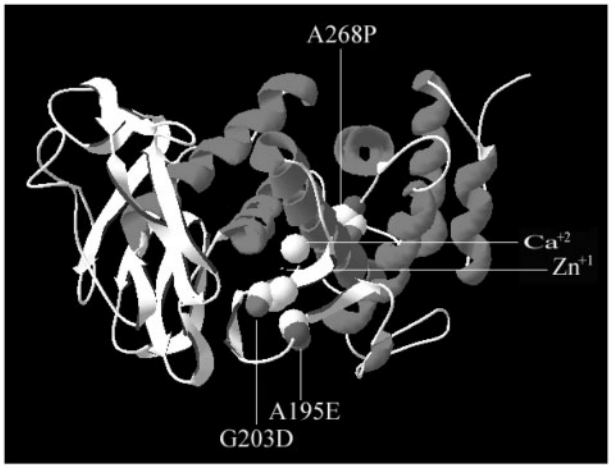

Fig. 1 Three-dimensional model of SVP was built with Modeller, using crystal structure of elastase from PAE as template. Positions of the substituted residues were shown. Big and small spheres represent Ca^{+2} and Zn^{+2}, respectively.

Q-Sepharose fast flow column. Active fractions were eluted at 0.15 M NaCl in 20 mM Tris/HCl buffer, pH 8.5, and finally loaded on Sephacryl S-200 gel filtration column. The purity of the enzymes was confirmed by SDS–PAGE (Fig. 2). In order to evaluate the secondary structural content of proteins, far-UV CD measurements were carried out. Compared to SVP and other mutants, A268P variant contains more secondary structures. Furthermore, the secondary structure of A195E and G203D variants are greater than SVP, while the secondary structure was slightly less in double mutant (Fig. 3a). SVP has five tryptophan residues located at positions 27, 191, 122, 249 and 266. Figure 3b shows the fluorescence spectrum of SVP and the other mutants in aqueous media. The maximum fluorescence intensity for SVP and TLN was observed at 340 nm and 337 nm, respectively, indicating that tryptophan residues in SVP are located in a more polar environment than TLN. The maximum intensity of A268P is similar to that of SVP protein, but A195E and A195E/A268P variants exhibit a red shift of ~10 and 15 nm, respectively. Modelling data suggest that A195E substitution is located close to Trp191 residue. Increased negative charge on this position increases polarity and may affect the microenvironment, thereby causing Trp 191 to be placed in the more polar environment. In porcine fructose-1,6 bisphosphatase, a red shift was attributed to the negative charge of Asp127 in contact with the indole side chain of Trp 57 (*34*). The catalytic activities of SVP and variants were investigated towards FAGLA. As shown in Table I, K_m of these mutants was decreased, whereas k_{cat} and catalytic efficiency increased. In order to consider the effects of water-miscible organic solvents on the behaviour of the enzyme, two distinct phenomena were considered: (i) the enzyme catalytic power when the solvent is incorporated into reaction medium, termed activity and (ii) the residual enzymatic activity after incubation for different lengths of time in a water/solvent mixture, termed stability (*35*). Based on the previous reports (*31*, *36*, *37*), different organic

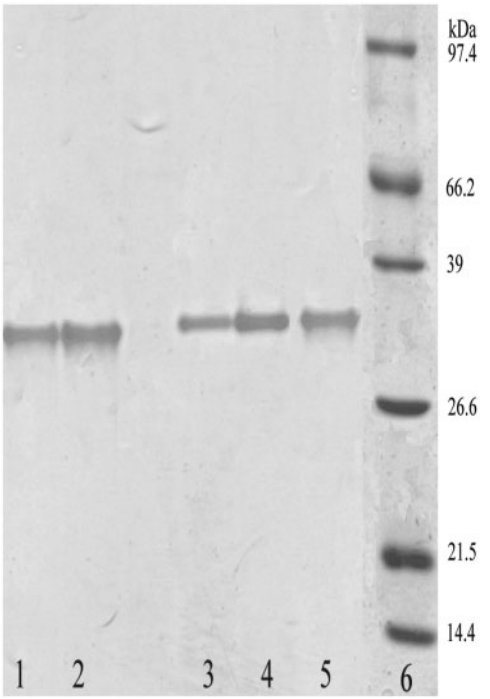

Fig. 2 SDS–PAGE of the purified SVP and mutants. SVP (lane 1), A195E (lane 2), G203D (lane 3), A268P (lane 4), A195E/A268P (lane 5) and protein markers (lane 6).

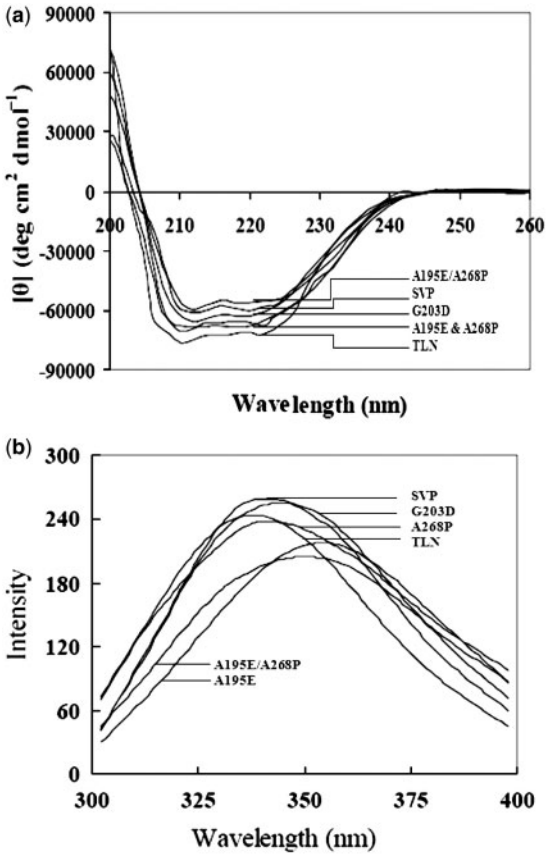

Fig. 3 The effect of substitutions on the far-UV CD and fluorescence spectra of SVP in aqueous solvent. (a) Far-UV CD spectra of TLN, SVP and mutants (A195E, G203D, A268P and A195E/A268P). An enzyme concentration of 0.2 mg ml^{-1} was used. (b) Fluorescence spectrum of TLN, SVP and mutants (A195E, G203D, A268P and A195E/A268P) with an enzyme concentration of 20 μg ml^{-1} in 20 mM Tris buffer, pH 7.

solvents (DMF, methanol, isopropanol and *n*-propanol) were selected for the investigation. These organic solvents have log *P* −1.0, −0.76, 0.14 and 0.28, respectively.

Protease activity of SVP and mutants in organic solvents

Protease activity was determined in the medium containing up to 40% (V/V) DMF, methanol, isopropanol and *n*-propanol (Fig. 4). The relative protease activity of TLN, SVP and its variants decreased with increasing concentrations of organic solvents. This phenomenon also occurred when other enzymes were incubated in the presence of organic solvents (*37–39*). Solvent concentrations where 50% of enzyme activity remains (C_{50}) are summarized in Table II. TLN exhibited low proteolytic activity

Table I. Kinetic constants of wild-type and mutants SVP.

	Kinetic parameters[a]		
Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} s^{-1}$)
SVP	0.406 ± 0.015	121 ± 1	298
A195E	0.301 ± 0.004	204 ± 2	677
G203D	0.315 ± 0.011	174 ± 1	552
A268P	0.381 ± 0.01	145 ± 1	381
A195E/A268P	0.361 ± 0.005	190 ± 1	515

[a]The kinetic values are the averages of three independent experiments and standard errors are <7%.

Table II. C_{50} values[a] of TLN, SVP and its variants in organic solvents.

	C_{50} (%, V/V)			
	DMF	Methanol	Isopropanol	*n*-propanol
TLN	10.5	16.5	6	4
SVP	11.2	17.5	6.5	4.8
A195E	15.4	22.5	9	7
G203D	14	21	8	6.3
A268P	12	18.5	7.3	5.8
A195E/A268P	13.2	20	7.3	5

[a]C_{50} is the value of the solvent concentration where 50% of enzyme activity remains. Relative C_{50} could be determined from this equation [(C_{50} value of mutant − C_{50} value of SVP)/C_{50} value of SVP] × 100.

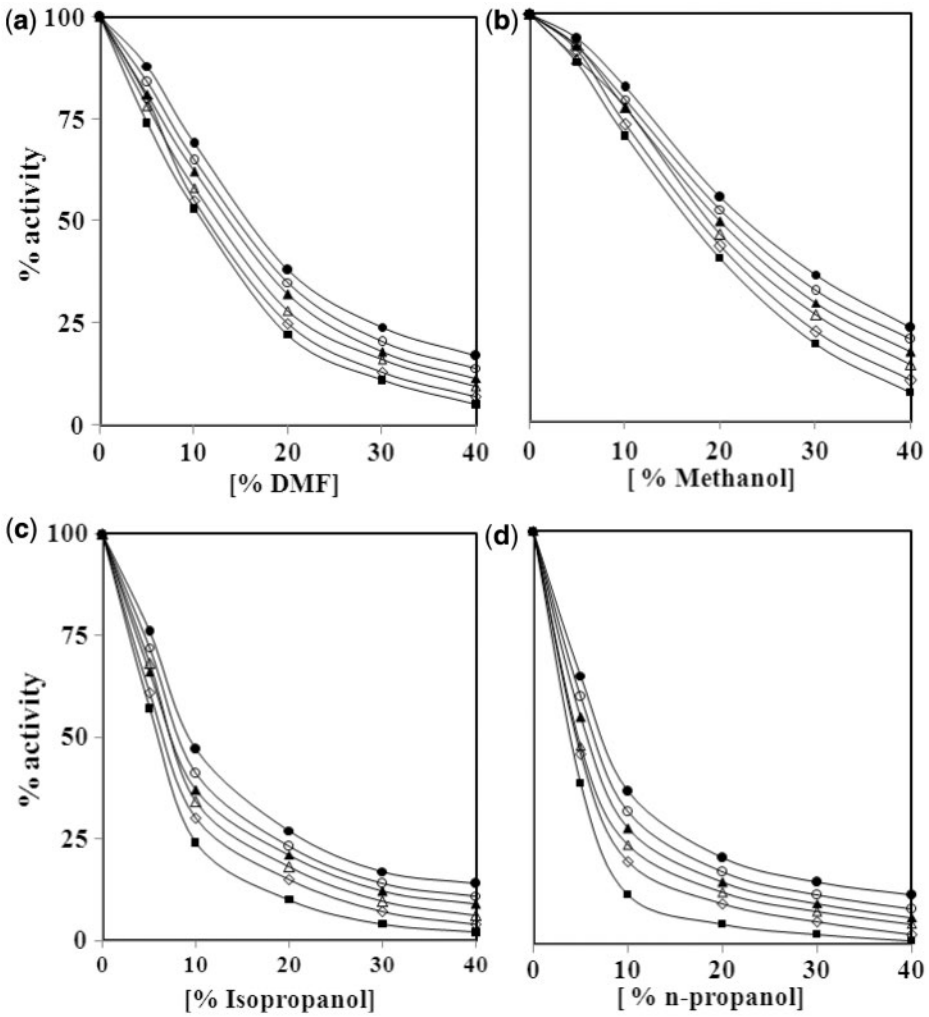

Fig. 4 Activity of TLN (filled square), SVP (open diamond) and mutants [A195E (filled circle), G203D (open circle), A268P (open triangle) and A195E/A268P (filled triangle)] at different concentrations of organic solvents: (a) DMF, (b) methanol, (c) isopropanol and (d) propanol. Different concentrations of organic solvents were prepared in 50 mM Tris, pH 7.0. Standard deviations were within 6% of experimental values.

compared to SVP and its variant. Since SVP is more active it is suitable for synthetic biotechnology uses.

Beside higher relative C_{50} values of A195E variant in DMF and methanol (26 and 11%, respectively), this mutant also demonstrated higher relative C_{50} value in isopropanol and *n*-propanol (32 and 41%, respectively), compared to SVP. C_{50} values estimated for G203D variant were slightly lower than A195E in the presence of organic solvents. Since polar and charged residues are sites of bound water and organic solvents tend to strip water from these sites, increased protease activity in A195E and G203D indicates an improvement in electrostatic interactions of the substrate-binding pocket that may increase water-binding sites and preserve hydration layer during catalysis. A268P was an effective substitution, which increased protease activity in the presence of isopropanol and *n*-propanol (Fig. 4 and Table II). It indicates that stabilization of the surface loop far from the active site has positive effects on the protease activity. The relative C_{50} values of double mutant (compared to SVP) were 6.6 and 5.8% in DMF and isopropanol. These results suggest an increase in the water-binding sites on the surface of enzyme in the substrate-binding pocket which stabilize the surface loop far from the active site that can improve protease activity in the presence of organic solvent.

DMF and methanol have lower log *P* than isopropanol and *n*-propanol; therefore, these solvents have more tendencies to strip water from the essential layer, thereby inactivating or denaturing the biocatalyst. Protease activity strongly decreases in the presence of isopropanol and *n*-propanol in spite of the higher log *P* for isopropanol and *n*-propanol, compared to DMF and methanol. Our previous results showed that the protease activity of TLN strongly decreases in isopropanol and *n*-propanol without any change in the tertiary structure of the enzyme. We assume that protease activity was inhibited in the low concentrations of mentioned organic solvents (*30*). The results show that the inhibition strength of the solvents increased with increments in organic solvent hydrophobicity.

Thermal inactivation of SVP and mutants in organic solvent media

The protease stability in the presence of organic solvents is an important factor when catalysing synthetic reactions. In the case of proteases, TLPs display irreversible unfolding at elevated temperatures because of autodigestion (*40*, *41*). The parameter often used to measure their stability is the rate of irreversible thermoinactivation (*41*, *42*). Irreversible thermal inactivation could be a complicated function of susceptibility to autodigestion, temperature and enzyme unfolding. Autolysis is known as the major mechanism responsible for irreversible inactivation of TLPs (*43*). It is interesting to analyse the stabilities of SVP and variants at even higher temperatures in the presence of organic solvent (*35*). The rates of irreversible thermoinactivation (k_i) of SVP and mutants were compared at 60°C in the presence of 40% (V/V) organic solvent (Fig. 5, Table III). The results showed that TLN is

Table III. The irreversible thermoinactivation[a] rate (k_i) of SVP and mutants.

	k_i ($\times 10^{-3}$ min^{-1})			
	DMF	**Methanol**	**Isopropanol**	***n*-propanol**
SVP	90 ± 5	60 ± 4	100 ± 3	190 ± 5
A195E	60 ± 3	48 ± 2	77 ± 2	130 ± 4
G203D	70 ± 2	50 ± 2	80 ± 5	160 ± 5
A268P	40 ± 5	20 ± 3	50 ± 5	90 ± 3
A195E/A268P	50 ± 4	30 ± 5	60 ± 3	120 ± 4

[a]Purified proteases were incubated in 50 mM Tris–HCl, pH 7, containing 40% (V/V) of organic solvent at 60°C for differing times. In the assay solution, the final concentrations of organic solvent and enzyme were 4% (V/V) and 20 μg ml^{-1}, respectively. Plots of the log of residual protease activity versus time were linear, indicating a first-order decay process under these conditions. No decrease in the activity of TLN was observed at this temperature.

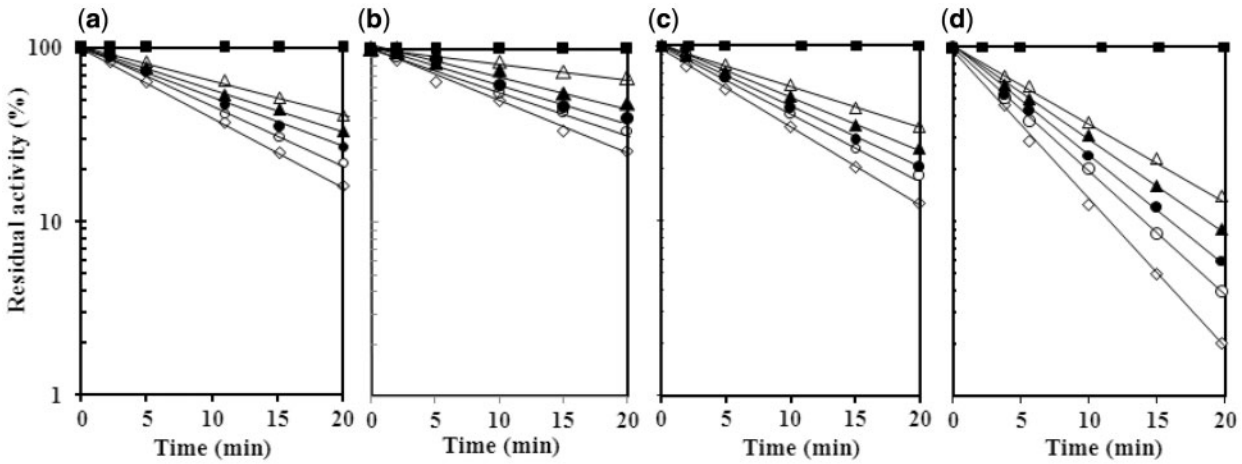

Fig. 5 Thermal inactivation of enzyme variants at 40% (V/V) of (a) DMF, (b) methanol, (c) isopropanol and (d) propanol. TLN (filled square), SVP (open diamond) and mutants [A195E (filled circle), G203D (open circle), A268P (open triangle) and A195E/A268P (filled triangle)] was incubated at 60 °C and at regular intervals, samples were picked up and cooled on ice and their residual protease activities were determined. Standards deviations were within 5% of experimental values.

highly stable in organic solvents. In contrast, TLN drastically lost its activity in isopropanol and *n*-propanol (Fig. 4). The results showed that A268P and A268P/A195E marginally increased the thermoresistance of the enzyme in this condition.

As shown in Table III, the k_i values of A195E and G203D variants decreased in all organic solvents, compared to SVP. These results indicate that increased negatively charge residues in the substrate-binding pocket did not only increase the protease activity (Fig. 4), but also decreased the thermal inactivation rate in the presence of organic solvents (Fig. 5, Table III). In comparison to SVP, k_i for A268P decreased ~2-fold in the presence of DMF, isopropanol and *n*-propanol. Furthermore, k_i calculated for A268P was found to be 20 ($\times 10^{-3}\,min^{-1}$) in the presence of methanol, while k_i for SVP in the same condition was estimated 60 ($\times 10^{-3}\,min^{-1}$) (Table III). These results indicate that stabilizing protein surface loops improve the enzyme stability in the mixture of water–miscible organic solvents. The value of this improvement depends on the nature of organic solvent. A195E/A268P variant exhibited 1.6- to 2-fold reduction in k_i compared to SVP in the presence of these organic solvents.

In the other experiment, TLN, SVP and its variants were incubated in 40% of various organic solvents (DMF, methanol, isopropanol and *n*-propanol) at 30°C with constant shaking for 15 days (data not shown). These results agreed well with those obtained from thermal inactivation of SVP and mutants in organic solvent media. In addition, results revealed that, TLN, SVP and some variants showed a remarkable increase in protease activity in the primitive hours of incubation. The same phenomenon was also observed for the other enzymes in the presence of organic solvents (*44*).

As mentioned previously, protease activity and stability in the presence of organic solvents can be improved by introducing negative surface-charges and stabilizing the surface residues, but it depends on the nature of organic solvent and substitution. The previous studies showed that activity and stability of enzymes in the presence of organic media have different characteristics (*45*). Some attempts to improve enzyme activity and stability in the presence of organic solvents were made using methods based on protein engineering. Most of the enzymes obtained using these methods were, however, still not stable in organic solvents, but a similar approach is probably useful for obtaining organic solvent stable enzymes (*45*). Random mutagenesis on protein engineering was performed by several investigators (*9*–*16*). The effective mutations were local near the substrate-binding pocket and/or the active site. For example, although these substitutions enhanced the activity of subitilisin E in the presence and absence of DMF, the stability of the mutant was slightly lower than that of the wild type (*9*, *11*). Arnold and coworkers showed that Q103R substitution created by random mutagenesis in subtilisin exhibited higher activity in the presence of DMF and water and had no deleterious effect on subtilisin stability (*11*). This mutation lies near the substrate-binding pocket. Stabilizing electrostatic integrations such as this could increase the substrate-binding affinity and therefore lower K_m. N218S also increased both stability and activity, but the stability of Q103R/N218S is identical to that of N218S (*11*). Chen and Engel engineered a phenylalanine dehydrogenase mutant, L307V, from *Bacillus sphaericus* with high activity and stability in organic–aqueous solvent mixtures (*46*). The fact that activity and stability are not incompatible in an enzyme has been described in some reports (*47*, *48*). Our results indicate that increased water bound sites on the surface of the enzyme in the substrate-bounding pocket can improve the protease activity and stability in the presence of organic solvent. However, it is related to the substitutions and nature of organic solvents.

Conclusion

To our knowledge, this is the first site-directed mutagenesis study that considers protease surface loops in the presence of organic solvents. A195E and G203D are located on the surface loops, close to the active site. The results indicate that increase water bonding sites, active site polarity and electrostatic interactions in the loops surrounding SVP active site may improve enzyme activity and stability in the organic solvent media. In addition, this study showed that increasing the rigidity of the loop far from the SVP active site may improve protease activity and stability in the presence of organic solvents. These mutants decreased the SVP thermal inactivation rate in the presence of all organic solvents. Overall, the current study suggests a strategy to develop new organic solvent-efficient enzymes by using site-directed mutagenesis.

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Conflict of interest
None declared.

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