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Protein Engineering of a Metalloprotease in Order to Improve Organic Solvents Stability and Activity

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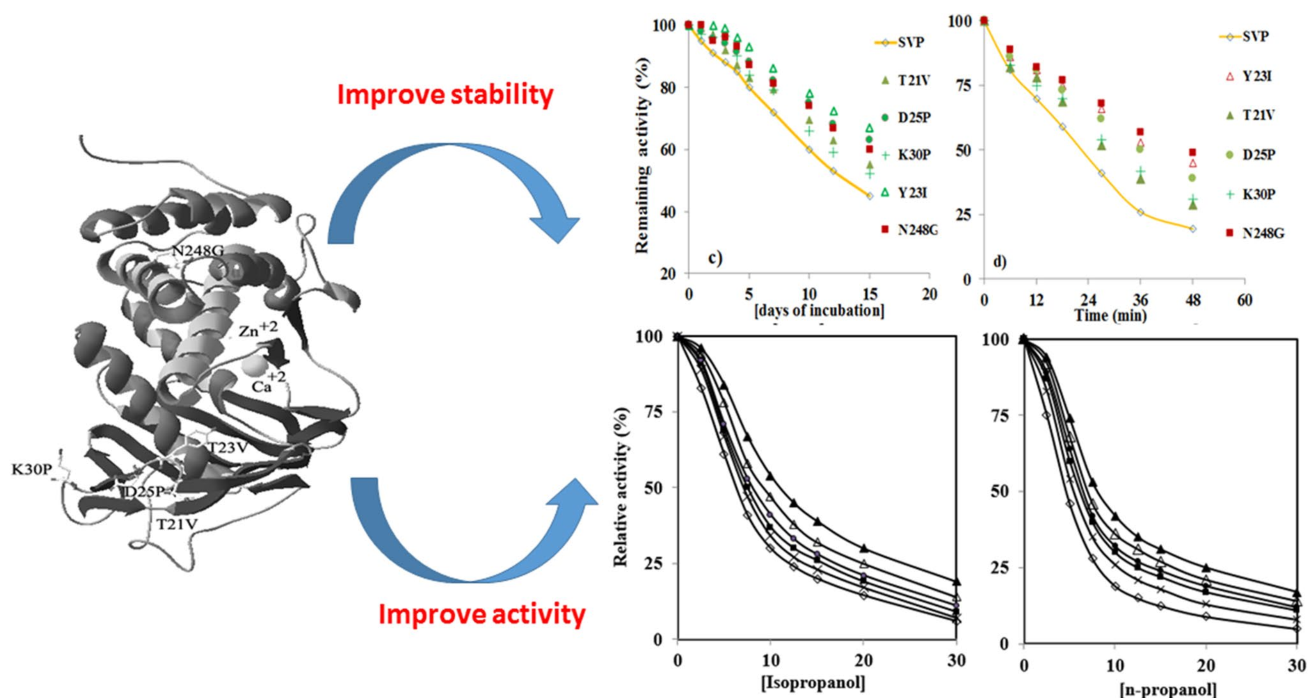
Abstract

Recently, improve the protease activity in the presence of organic solvents has been appreciated for the researchers. In the current study, we have tried to increase the organic solvent stability of *salinovibrio proteolyticus* protease (SVP) by site-directed mutagenesis. Five variants were constructed to substitute the surface charged, and polar amino acid residues in SVP with hydrophobic ones (T21V, Y23V, K30P, D25P and N248G) to examine the outcome of surface hydrophobicity on the enzyme efficiency in the presence of organic solvent. The catalytic efficiency of Y23V and N248G mutants not only increased about 1.8 and 2.6 folds in DMF and methanol but also increased it about 3.8 and 5.0 folds in isopropanol and n-propanol, compared to SVP. $\Delta\Delta G^{\ddagger}$ values of Y23V and N248G variants, increased about 6.5 and 9.5 kcal mol^{-1} in DMF and methanol, and it improved about 13.6 and 16.6 kcal mol^{-1} in isopropanol and n-propanol, respectively. These results show that irreversible thermoinactivation rate of protease has a straight relationship with hydrophobicity of organic solvents.

Graphic Abstract



Keywords Site-directed mutagenesis · *Salinovibrio proteolyticus* protease · Catalytic efficiency · Organic solvent

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1 Introduction

The ability to use enzymes in organic solvents expands the potential applications of biocatalysts in chemical transformations which is useful for many industries [1, 2]. Poor stability and low catalytic activity of enzymes are the limitations of using enzymes in organic solvents [3, 4]. Regrettably, Mother Nature has not intended enzymes to perform in polar organic solvents, which regularly act as robust denaturants and rapidly deactivate enzymes. Non-aqueous solvents molecules bond to polar amino acid residues, and thus water-striping phenomena from an enzyme does happen and enzyme activity reduces [5–8]. Some efforts to advance the enzyme activity and stability in the presence of organic solvents were prepared to expend approaches based on enzyme engineering. Most of the obtained biocatalysts using these approaches were not appreciated stable in organic solvents. Nonetheless an analogous methodology is possibly valuable for gaining the organic solvent tolerant bio-catalysts [9]. Random mutagenesis of subtilisin, chloroperoxidase and phospholipase A_1 based on protein engineering has been accomplished by numerous researchers [10–16]. Furthermore, there are some reports in the literature regarding the site directed mutagenesis (SDM) of the enzyme in the presence of organic solvents [17–20].

Salinivibrio zinc-metalloprotease (SVP) is a member of the family of thermolysin-like proteases (TLPs) [21]. One of their features is a zinc ion which presented in the active site of SVP protease. Thermolysin-like proteases contain a C-terminal domain with alfa-helical structure and a beta-rich N-terminal domain. These two domains coupled by a principal alfa-helix, which positioned at the end of the active site split and which encompasses numerous catalytically significant amino acid residues. In the databases, this SVP protease is the earliest amino acid sequences of bacterial proteases which obtained from temperately halophilic bacteria. SVP protease is active at alkaline pH (optimal 8.5–10), and over an extensive variety of salt concentrations (0.0–4.0 M) [22–24]. Stability in high salt concentration and alkaline pH are the most important features for the protease applications in the synthetic biotechnology [25]. Consequently, improve the stability and efficiency of this SVP protease has been appreciated for the synthetic biotechnology.

In our previous results, we rationally engineered surface-charged residues (A195E and G203D) of SVP protease near the substrate-binding region and active center based on results of directed evolution and crystallographic of proteases in organic solvents [26]. Our results displayed that, the enzyme activity and stability in non-aqueous organic solvents developed with growing active site polarity of SVP by site directed mutagenesis. A268P variant (stabilize an external loop adjust to the active site of enzyme) and A268P/A195E variant slightly improved the thermo-tolerant of the enzyme [26]. In the present study, five surface accessible variants were designed to substitute the surface charged, and polar amino acid residues in SVP with hydrophobic ones (T21V, Y23V, K30P, D25P and N248G) to examine the outcome of surface hydrophobicity on the enzyme efficiency in aqueous and organic solvent media.

2 Materials and Methods

2.1 Materials

Primers manufactured by Bioneer Company (South Korea). PWO DNA Polymerases acquired from Roche (Germany). Restriction enzymes and PCR reagents gained from Fermentase (Germany). FAGLA (3-(2-furylacryloyl)-L-glycyl-L-leucine-amide) was purchased from Bachem Company (Germany). Organic solvents were all in analytical evaluation. Reproducibility of the data established by repeating the test at least in triplicate. The investigational error was not ever over 7%.

2.2 Modeling and Mutation Design

A three-dimensional classical of SVP was assembled based on the identified structure of elastase from *P. aeruginosa* (PAE) (PDB code 1EZM) [27] which conserves the general fold shared by the other neutral metalloproteases (thermolysin from *B. thermoproteolyticus* [28] and neutral metalloproteases (NPs) of *B. cereus* [29]. Modeling and mutation design was performed based on our previous reports [26, 30]. For the modeling method, the sequence of SVP was acquiesced to the Swiss Model system, using the first methodology approach in blend with the DeepView 3.7 program. Among ten created models for each structure, the structures with the lowest unbiased function selected [26]. Surface available residues of SVP determined with ASA View. ASA View is a database and web server for the graphical demonstration of solvent availability of a protein. In addition, our previous report indicated that proline had important role in improving organic solvent stability. Site directed mutagenesis of SVP achieved by using the technique designated by Fisher and Pei [31]. The PCR reaction performed based on our previous reports [30]. The primers were planned as follow:

Thr 21 Val forward: 5- CGGGTCAATATCTGTATGGCG TGGATTATGATGACTTCCCCG -3

Thr 21 Val reverse: 5- CGGGGAAGTCATCATAATCCA CGCCATACAGATATTGACCCG-3

Tyr 23 Val forward: 5- CTGTATGGCACCGATGTGGAT GACTTCCCCGTCG -3

Tyr 23 Val reverse: 5- CGACGGGGAAGTCATCCACAT CGGTGCCATACAG -3

Asp 25 Pro forward: 5- GTATGGCACCGATTATGA TCCGTTCCCCGTCGATAAAGTGG -3

Asp 25 Pro reverse: 5- CCACTTTATCGACGGGGA ACGGATCATAATCGGTGCCATAC -3

Lys 30 Pro forward: 5-GATGACTTCCCAGTCGATCCG GTGGGCGATGTGTGTACC-3

Lys 30 Pro reverse: 5-GGTACACACATCGCCCACCGG ATCGACTGGGAAGTCATC-3

Asn 248 Gly forward: 5-CTCCTTGCTAACAAGCCA GGCTGGGATGTCCGCAAAGG-3

Asn 248 Gly reverse: 5-CCTTTGCGGACATCCCAG CCTGGCTTGTTAGCAAGGAG-3

2.3 Expression and Purification of SVP and Mutants

The SVP protease gene was highly expressed using pQE-80L plasmid [23]. The fabricated expression vector was used to transform into *E. coli* strain BL21 (DE3). Colonies booming SVP protease genes were cultured in 400 ml of LB medium inoculated with ampicillin at 37 °C, 224 rpm. 1 mM concentration of IPTG was supplemented, and then cultivated for 24 h. The bacterial cells were centrifuged at 8000×*g* for 15 min at 4 °C, and lastly the supernatant having active protease was concentrated by an ultrafiltration method.

The SVP protease was moderately purified in one phase on Q-Sepharose chromatography column (1 × 10 cm) which equilibrated with 20 mM Tris–HCl buffer, pH 8.5. The obtained SVP protease was lastly loaded on a Sephacryl S-200 column (1 × 100 cm), which previously equilibrated with the mentioned buffer. The purity of SVP and mutants was evaluated using 12.5% SDS-PAGE [30]. Protein amount was also calculated rendering to the procedure of Bradford using bovine serum albumin (BSA) as the standard.

2.4 Enzyme Assay in Aqueous and Non-aqueous Organic Solvent

Protease assay was also investigated by expending FAGLA, as an artificial substrate of TLPs (Thermolysin-like proteases) [32–34]. Hydrolysis of the mentioned substrate by SVP and constructed mutants was dignified succeeding the reduction in absorbance at 345 nm [35–37]. The quantity of substrate hydrolyzed was calculated using the molar absorption alteration owing to hydrolysis, $\Delta\varepsilon_{345} = -310\ M^{-1}\ cm^{-1}$, at 25 °C. The protease activity and calculation of the variations in transition-state stabilization energies ($\Delta\Delta G^{\ddagger}$) were performed based on our previous paper [30].

For considering the protease activity in the presence of organic solvents, the mixture of water and organic solvent prepared, and protease activity value was considered in every test [38]. After making the varied concentrations of organic solvent, the pH was measured and attuned to the necessary amount. The outcome of increasing the non-aqueous solvent value up to 30% (V/V) was explored in the standard assay situation as defined above. Activity is expressed as the remaining protease activity relative to control without any organic solvent (100%). Eight points (0, 2.5, 5, 7.5, 10, 15, 20, and 30%) of different organic solvents have been elected between 0 and 30% to consider the protease activity in the presence organic solvents. C_{50} is the amount of the solvent amount where 50% of protease activity remnants. Kinetic parameters for SVP and mutants explored from a sequence of original rates at the diverse amount of FAGLA substrate (0.01 to 3.0 mM) in aqueous solvent and in the presence of 10% (V/V) organic solvents. All quantities accomplished three times.

2.5 Thermal Inactivation in the Presence of Organic Solvent

Purified proteases in Tris/HCl buffer containing 40% (V/V) of organic solvent maintained at 60 °C for varied time intervals [13, 26]. At subsequent time interval, fresh samples were picked up, placed on ice and then the remaining enzyme activity was measured. In the assay reaction, the final amount of non-aqueous solvent and protease were 4% (V/V) and 20 µg/ml, respectively. The protease activity of the blend of enzyme/organic solvent that retained on ice measured as control (100%) [26]. Plots of the log of remaining protease activity against time were linear, signifying a first-order decay procedure under these situations. So, the speed of irreversible thermal inactivation (k_i) of SVP and mutants considered.

2.6 Organic Solvent Stability

The enzyme stability in the presence of the mentioned organic solvents was investigated by incubating the purified SVP and variants with organic solvent at 30 °C with 160 rpm for 15 days [9, 39]. Final concentration of organic solvent in the incubation reaction was 40% (v/v). Immediately after the adding of the organic solvent, at different time intervals (1, 2, 3, 4, 5, 7, 10, 12, 15 days), 50 µl of each samples were considered for the remaining enzyme activity. The final concentration of each organic solvent and the enzymes (SVP and variants) in assay reaction were 4% (V/V) and 20 µg/ml, respectively.

2.7 Structural Studies

Fluorescence quantities were performed using a Spectrophotometer with the protease value of 30 µg/ml in 25 mM Tris buffer, pH 7.5. The components were blended and permitted

to equilibrate for 5 min. The wavelength of excitation was 280 nm, and the emission fields documented from 300 to 400 nm. Circular dichroism (CD) tests were performed on Aviv model 215 CD spectrophotometer consuming of 20 mM Tris, pH 7. Results are stated as molar ellipticity [θ] (deg cm^2 dmol^{-1}), and it was considered from the formula $[\theta]_\lambda = (\theta \times 100\mathrm{MWR})/(cl)$, where c is the protein amount in mg/ml, l the light path span in centimeters, and θ is the dignified ellipticity in grades wavelength λ.

3 Results and Discussion

3.1 Mutants Design

Previous reports have revealed that the substitution of the moderately buried polar residues on the surface of a protein with hydrophobic amino acid residues can lead to an improvement in the protein stability [40]. Also, studies by Kawata and Ogino [41] shown that the constructing growing forces in the protein structure lead to an increase of enzyme stability in the organic solvent media. Monsef-shokri and co-workers (2013) reported that, the increasing hydrophobic patch on the lipase surface could assistance to have a more stable enzyme in organic solvent [42]. To select the greatest residues for such strategy, ''ASAview'' server was used to explore the surface residues and their surface accessibility. According to universal rules for the mutation design in organic media, non-conservative amino acid residues that are not involved in the fatal structural elements or in any serious intra-molecular interactions, while being far away from the active site are suitable candidates for the mutation [43]. Therefore, we substitute the surface charged and polar amino acid residues in SVP with hydrophobic ones (T21V, Y23V, K30P, D25P and N248G) to examine the outcome of surface hydrophobicity on the protease efficiency in aqueous and non-aqueous solvent media.

3.2 Biochemical Characterization in the Aqueous Solvent

Fluorescence spectra exhibited that no noteworthy alteration between these mutants and SVP was observed in the aqueous solvent. Even though, all variants displayed the similar λ_{max} as SVP; but, N248G variant exhibited more fluorescence intensity in which T21V, D25P and K30P show lower fluorescence intensity (Fig. 1a). Far-UV CD studies showed that in D25P and K30P variants, the secondary structure marginally increased in the aqueous solvents related to SVP (Fig. 1b). Generally, the results indicated that, the exchanging surface charged and polar amino acids with hydrophobic ones in SVP cannot prompt conformational fluctuations.

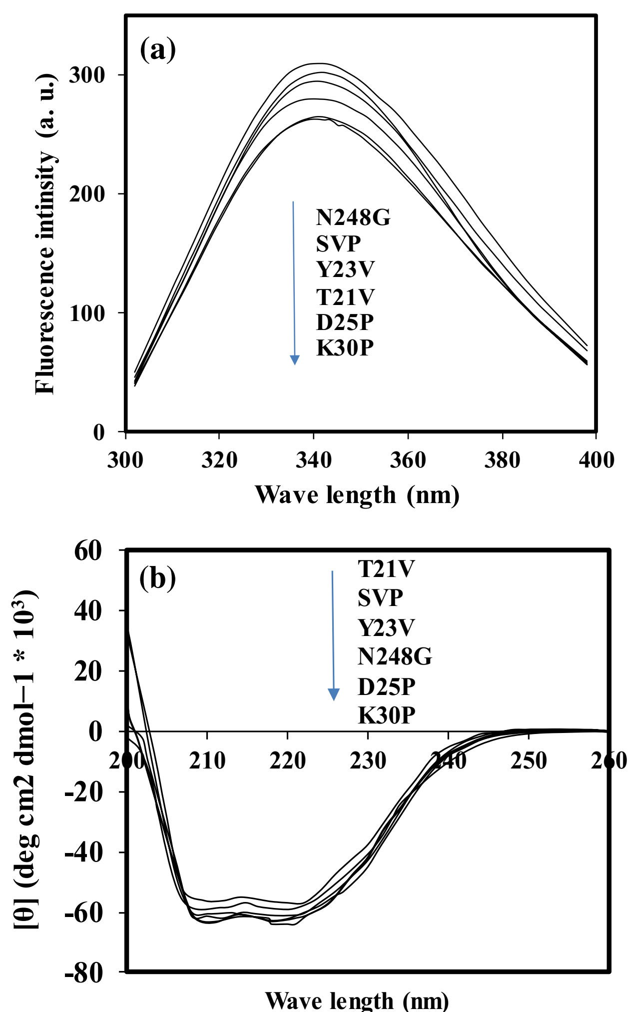

Fig. 1 **a** Fluorescence spectrum of SVP and mutants with enzyme concentration of 20 µg/ml in 20 mM Tris buffer, pH 7 in the aqueous solvent. **b** Far-UV circular dichroism spectra of SVP and mutants in aqueous solvent. An enzyme concentration of 0.2 mg/ml was used

To explore the outcome of these replacements on the enzyme activity in aqueous solvent, kinetic factors were calculated using FAGLA as synthetic substrate, and the data were presented in Table 1. In aqueous solvent, T21V and Y23V decrease kcat, but, kcat value of D25P, K30P and N248G mutants slightly increased, compared to SVP. In which, Y23V variant is the only mutant that it,s K_m decrease about 0.1 mM, compared to SVP.

Also, N248G variant is the only mutant that increased catalytic efficiency about 1.25 fold, and diminish the free energy of transition-state stabilization ($\Delta\Delta G^{\ddagger}$) about 2.26 kcal mol^{-1}. It is mention that, T21V variant decreases $\Delta\Delta G^{\ddagger}$ about 2 kcal mol^{-1}, but N248G variant increase it about 2.26 kcal mol^{-1}. The current results are similar to the kinetic data of substrate hydrolysis.

Table 1 Kinetic constants of SVP and variants

	SVP	T21V	Y23V	D25P	K30P	N248G
Aqueous solvent						
*K*m (mM)	0.406 ± 0.015	0.416 ± 0.004	0.310 ± 0.002	0.413 ± 0. 004	0.439 ± 0.005	0.371 ± 0.001
*k*cat (S^{-1})	121 ± 1	102 ± 1	102 ± 1.5	133 ± 1.5	135 ± 0.81	138 ± 0.5
*k*cat/Km	298	245	329	322	307	372
$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})		+ 2.01	− 1.04	− 0.8	− 0.30	− 2.26
DMF						
*K*m (mM)	0.844 ± 0.006	0.83 ± 0.005	0.71 ± 0.005	0.846 ± 0.005	0.821 ± 0.008	0.68 ± 0.001
DMF *k*cat (S^{-1})	39 ± 0.17	52 ± 1.12	61 ± 1.18	53 ± 0.81	44 ± 0.75	61 ± 0.32
*k*cat/Km	46	62	86	62	53	90
$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})		− 2.20	− 6.50	− 2.88	− 1.56	− 6.82
Methanol						
*K*m (mM)	0.554 ± 0.016	0.44 ± 0.01	0.4 ± 0.013	0.53 ± 0.007	0.58 ± 0.007	0.48 ± 0.011
Methanol *k*cat (S^{-1})	56 ± 0.14	80 ± 0.11	102 ± 0.1	90 ± 0.77	96 ± 0.18	128 ± 0.1
*k*cat/Km	101	182	255	170	165	266
$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})		− 6.00	− 9.45	− 5.03	− 5.01	− 9.88
Isopropanol						
*K*m (mM)	0.453 ± 0.001	0.44 ± 0.002	0.43 ± 0.001	0.489 ± 0.003	0.59 ± 0.003	0.39 ± 0.001
Isopropanol *k*cat (S^{-1})	38 ± 2	59 ± 2	137 ± 2	88 ± 2	104 ± 1	125 ± 2
*k*cat/Km	84	134	318	180	176	320
$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})		− 4.84	− 13.62	− 7.78	− 7.55	− 13.66
n-Propanol						
*K*m (mM)	0.354 ± 0.003	0.34 ± 0.003	0.35 ± 0.003	0.38 ± 0.002	0.393 ± 0.003	0.321 ± 0.004
n-propanol *k*cat (S^{-1})	25 ± 1	54 ± 3	124 ± 4	74 ± 1	63 ± 2	120 ± 6
*k*cat/Km	70	159	354	195	160	374
$\Delta\Delta G^{\ddagger}$ (kcal mol^{-1})		− 8.50	− 16.58	− 10.46	− 8.50	− 17.14

The kinetic values are the averages of three independent experiments and standard errors are less than 10%

Final concentration of organic solvent in assay reaction was 30% (V/V)

Because of protease auto-digestion at high temperatures, thermolysin-like proteases exhibited irreversible denaturation at this condition [44, 45]. To explore the results of surface hydrophobicity on the enzyme stability, the level of irreversible thermoinactivation (k_i) of SVP and its mutants were examined at 60 °C (Table 2). Results indicated that T21V and Y23V variants increased k_i value about 1.2 and 1.4 ($\times 10^{-3}$ min^{-1}), respectively, but the other mutants

Table 2 The irreversible thermoinactivation[a] rate (k_i) of SVP and mutants

	k_i ($\times 10^{-3}$ min^{-1})				
	Increasing log P				
	No organic solvent	DMF (− 0.1)	Methanol (− 0.76)	Isopropanol (0.14)	n-propanol (0.28)
SVP	122 ± 4	94 ± 5	68 ± 5	104 ± 4	190 ± 5
T21V	148 ± 3	73 ± 2	57 ± 2	75 ± 1	155 ± 4
Y23V	174 ± 3	52 ± 3	40 ± 2	56 ± 2	106 ± 5
D25P	61 ± 2	68 ± 2	51 ± 3	72 ± 2	139 ± 4
K30P	110 ± 4	85 ± 2	60 ± 3	82 ± 3	170 ± 3
N248G	91 ± 5	60 ± 2	46 ± 2	64 ± 3	124 ± 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 assay solution, final concentration of organic solvent and enzyme were 4% (V/V) and 20 µg/ml, respectively. Plots of the log of remaining protease activity data versus time were linear, indicating a first-order decay process under these conditions. No decrease in activity of Thermolysin (TLN) was observed in this temperature

decrease it. D25P mutant is the only variant that decreases irreversible thermoinactivation rate about two times compared to SVP.

D150N and D150Q variants of thermolysin-like proteases from *B. stearothermophilus* eliminate the charge at this site and D150N demonstrates a 60% decrease in activity, while D150Q displays a 25% increase in protease activity. The charge was shifting at location 150 as in D150E caused in a 90% rise in protease activity at pH 6.8. In overall, these results indicated that the substitution of surface charged residues with hydrophobic ones lead to distribute the original hydrogen bonds and so cause the slight alterations in the three-dimensional structure of protein. These reformation in three-dimensional structure of protein may be effect on the accessibility of active site at the junction of N-terminal domain enriched β-sheet and C-terminal domain enriched α-helix.

Irreversible thermal inactivation could be a complicated function of susceptibility to autodigestion, temperature and enzyme unfolding. Thermolysin-like proteases display irreversible unfolding at elevated temperatures because of autodigestion [42, 43]. The most critical amino acids of the thermolysin-like proteinase (TLP) from *B. stearothermophilus* appear to be the phenylalanine residue at location 63 and the proline residue at location 69 [46]. Further changes at location 63 have revealed that the hydrophobic interaction among the phenyl ring of a phenylalanine residue at location 63 and the aliphatic portions of numerous surface residues in its situation (Val9, Arg11, Gln17, and Gln61) are significant [47]. In addition, the stabilizing result of proline residue at location 69 is possibly owing to its rigidifying result, which diminishes denaturation of the moderately unfolded constructions that are potential structures for auto-digestion [48, 49]. Some reports showed that the mutations in the C-terminal region have slight effect on the stability of TLN compared to mutation on the N-terminal region [48–50].

In overall, the previous reports indicated that the thermal tolerant of thermolysin-like proteases is principally determined by confined unfolding in the surface-located residues in the N-terminal region [51–55]. In spite of that, results showed that our mutations do not have the considerable effect on the thermal stability of SVP in the aqueous solvent. It is mentioned that our four substitutions (T21V, Y23V, D25P and K30P) are situated in the stability-determining area in the N-terminal region.

3.3 Enzyme Activity in the Presence of Organic Solvents

DMF, methanol, isopropanol, and n-propanol were chosen with the purpose of considering the outcome of mutations on the activity and stability of the enzyme in organic solvents [9, 56, 57]. Enzyme activity of SVP and its variants was considered in the reaction having up to 30% (v/v) DMF, methanol, isopropanol and n-propanol (Fig. 2). These results exhibited that protease activity of SVP diminished with the growing of the organic solvents concentration. The organic solvent concentrations where 50% of protease function were irreversibly (C_{50}) reduced in the order DMF > methanol > iso-propanol > n-propanol. DMF and methanol are polar non-aqueous solvents with log *P* of −0.1 and −0.76, respectively.

Water stripping from a protease into an organic solvent media does happen and can be important in polar solvents and they are capable of penetrating the protein inside, while nonpolar solvents cannot partition through the external polar surface of a protein [58]. Therefore, the protease activity diminished in the presence of DMF and methanol. Concerning this evidence, it appears that increasing the hydrophobicity or declining the water connecting positions can disturb the function in the polar non-aqueous solvents (DMF and methanol).

Isopropanol and n-propanol solvents are less polar organic solvents related to water which have log *P* about 0.14 and 0.28, respectively. Interruption of the native forces by these solvents (less polar than water) may lead to reduce the activity of SVP in the presence of isopropanol and n-propanol [26]. Our past data displayed that SVP was suppressed in the low amounts of these organic solvents [26, 38]. Outstandingly, bulk water is not completely essential and sometimes, as tiny as tens of H_2O molecules around the protein are satisfactory for noticeable enzyme activity [58, 59]. It suggested that the removing of water-binding sites can be a good rational approach for finding organic-tolerant enzymes. Protease activity consideration and kinetic factors of SVP and its mutants in the presence of organic solvents are discussed in the subsequent parts:

C_{50} is the amount of solvent where 50% of protease activity remnants and relative C_{50} were obtained from the following calculation:

(C_{50} value of each mutant- C_{50} value of SVP)/C_{50} value of SVP).

Relative C_{50} values as results shown in Fig. 3 and Table 3, C_{50} values of these variants increased in the organic solvent, in comparison to SVP. In addition, Y23V variant shows the most increase in relative C_{50} values in DMF and methanol about 36 and 39%, respectively and 83 and 78% in isopropanol and n-propanol, respectively. N248G variant also shows a good increase in the C_{50} values in the presence of organic solvents compared to SVP. Relative C_{50} of all mutants have more amount in the hydrophobic organic solvents than polar non-aqueous solvents.

Kinetic parameters results in Table 1 showed that compared to aqueous solvent; in organic solvents *K*m of native enzyme increase except for n-propanol. In most cases, decrease the enzyme activity is primarily due to a reduction of kcat and an increase in Michaelis–Menten constant *Km*

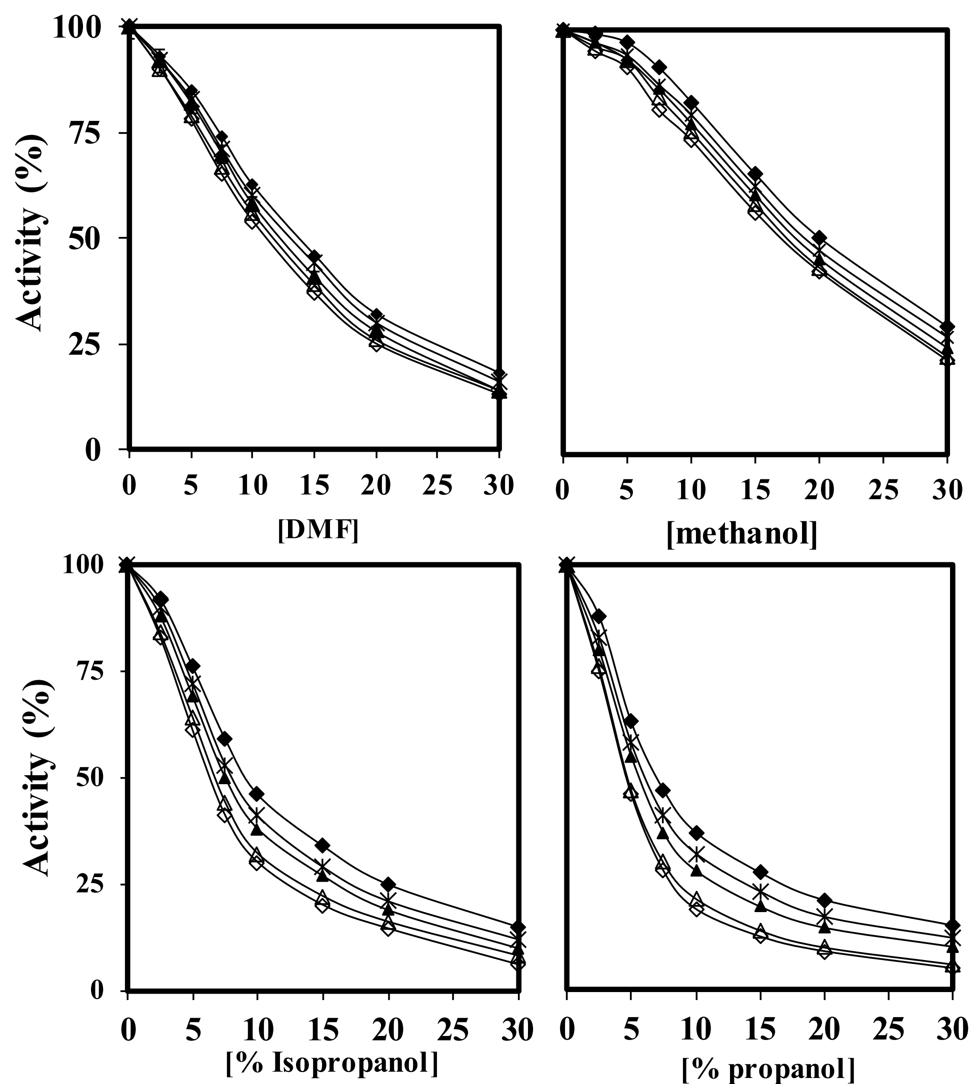

Fig. 2 Activity of SVP [open diamond] and mutants [T21V (filled diamond), Y23V (filled triangle), K30P [×], D25P (filled circle) and N248G (open triangle)] at different concentrations of organic solvents: DMF, methanol, isopropanol and propanol. Different concentrations of organic solvents were prepared in 50 mM Tris and subsequently pH of solutions was adjusted to 7.0

except for n-propanol. Catalytic efficiency strongly decreases in organic solvents. kcat values show more decrease in the hydrophobic organic solvent than polar ones. K_m of all variants increase in DMF, methanol, and isopropanol, compared to aqueous solvent.

In organic solvents, both kcat and kcat/*K*m parameters of all variants increase with the increasing of hydrophobic strength of organic solvents compared to SVP. The catalytic efficiency of Y23V and N248G mutants not only increased about 1.8 and 2.6 values in DMF and methanol, but also increased it about 3.8 and 5 values in isopropanol and n-propanol, compared to SVP. $\Delta\Delta G^{\ddagger}$ of all mutants improved in organic solvents, related to SVP. $\Delta\Delta G^{\ddagger}$ values of Y23V and N248G variants, strongly increased about 6.5 and 9.5 kcal mol^{-1} in DMF and methanol, respectively and it extraordinarily improved to 13.6 and 16.6 kcal mol^{-1} in the attendance of isopropanol and n-propanol (Table 2).

It was shown that the organic solvents molecules bond to the charged and polar amino acid residues and consequently water-striping from an enzyme in an organic solvent reaction does happen and activity reduces [5]. The solubility of substrate can alter the *K*m of enzymes in different solvent. Probably, the solubility of FAGLA, which used as substrate in this study, in the presence of different solvents can alter the *K*m of wild type and its mutants in DMF, methanol and isopropanol, except n-propanol. In addition, the probability of substrate solubility in these organic solvents may be increases the enzymes *K*m.

3.4 Thermal Inactivation in Organic Solvents

At higher temperature, TLPs are denaturized as a significance of autolysis. Auto-digestion tracks first-order kinetics since its speed is calculated by confined unfolding developments that reduce the protease vulnerable to auto-digestion cleavage [47, 48, 50, 60–62]. Irreversible thermo-inactivation rate (k_i) of SVP and its variants were related at 60 °C

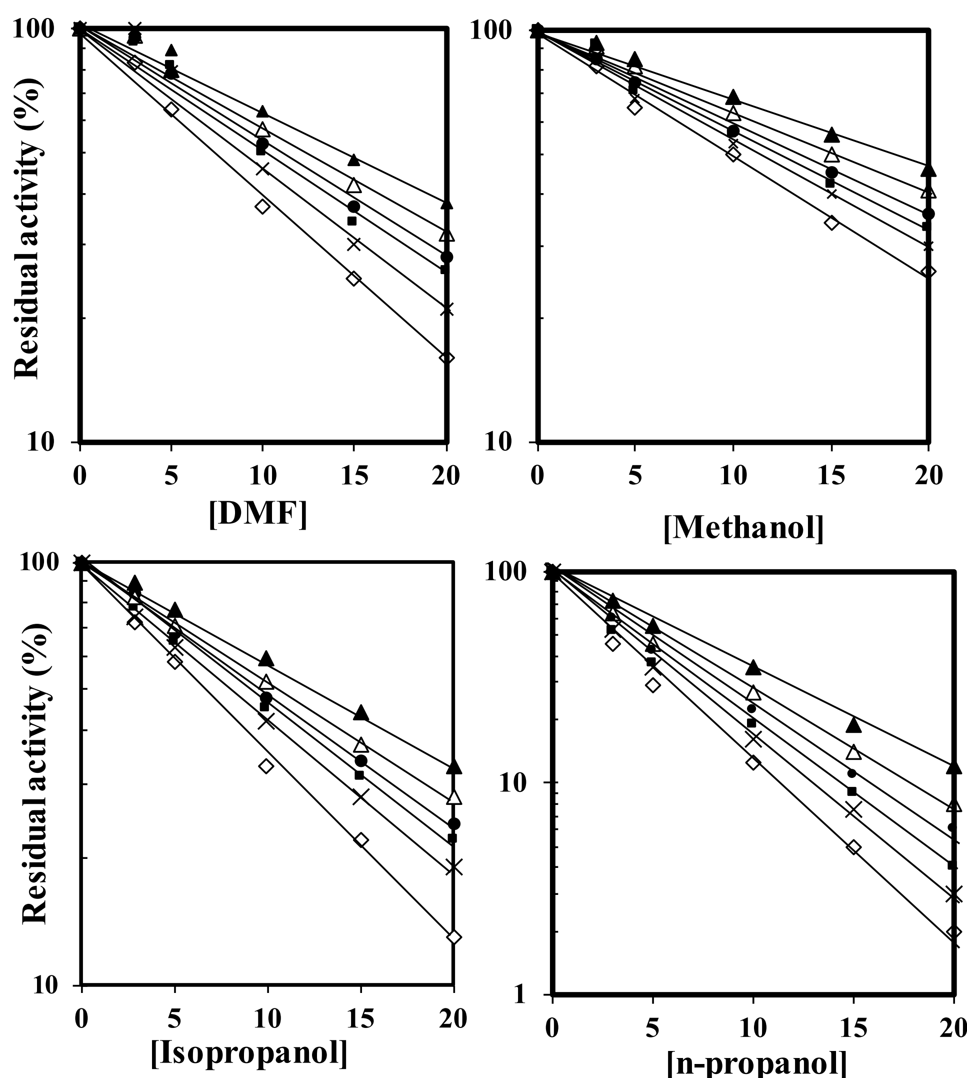

Fig. 3 Thermal inactivation of SVP [open diamond] and mutants [T21V (filled diamond), Y23V (filled triangle), K30P [×], D25P (filled circle) and N248G (open triangle)] in DMF (**a**), methanol (**b**), isopropanol (**c**) and n-propanol (**d**) with final concentration of 40% (V/V). The enzyme was incubated at 60 °C and at regular intervals, samples were removed and cooled on ice and their residual activities were determined as described in Sect. 2. Standards deviations were within 5% of experimental values

Table 3 C_{50} values of SVP and its variants in organic solvents

	C_{50} (%, V/V)			
	Increasing log P			
	DMF	Methanol	Isopropanol	n-propanol
SVP	11 ± 0.4	18 ± 0.5	6 ± 0.4	4.5 ± 0.3
T21V	12.5 ± 0.3	19.5 ± 0.2	7.5 ± 0.2	6 ± 0.1
Y23V	15 ± 0.2	25 ± 0.2	11 ± 0.4	8 ± 0.2
D25P	13 ± 0.1	22 ± 0.4	8 ± 0.3	6.5 ± 0.2
K30P	12 ± 0.2	21 ± 0.3	7 ± 0.2	5.5 ± 0.1
N248G	14 ± 0.3	23 ± 0.3	9.5 ± 0.2	7 ± 0.1

C_{50} is the value of the solvent concentration where 50% of enzyme activity remains

in the presence of 40% (V/V) organic solvent (Fig. 3 and Table 2). These results exhibited that in aqueous solvent, although k_i value calculated for T21V and Y23V mutants was higher than that for SVP, k_i value calculated for D25P, K30P and N248G mutants was lower than that for SVP.

D25P mutant decrease k_i value about two times compared to SVP in aqueous solvent. The observed reduction in the inactivation rate in the organic solvents compared to aqueous media might be due to the diminished auto-digestion, which itself is the consequence of the reduction in the activity of the enzyme in the organic solvents. In the subsequent portion, k_i value of SVP and mutants are discussed distinctly;

Results show that Y23V variant is the most effective mutant in this experiment because it shows the lowest k_i values between these mutations compared to SVP. It not only decreases k_i value about 42 and 28 ($\times 10^{-3}$ min^{-1}) in DMF and methanol but also decreases it about 48 and 84($\times 10^{-3}$ min^{-1}) in isopropanol and n-propanol. In addition, N248G is also a thermoresistance mutant in organic solvents that shows 34 and 22 ($\times 10^{-3}$ min^{-1}) in DMF and methanol, but it 40 and 66 ($\times 10^{-3}$ min^{-1}) in isopropanol and n-propanol.

Results show that k_i values of all mutants in n-propanol are 2 and 3 times lower than k_i value of them in DMF and methanol respectively, compared to SVP.

These results suggested that the rates of irreversible thermoinactivation are lower in the polar organic solvent than the hydrophobic organic solvent. These results show that irreversible thermo-inactivation rate has a straight relationship with the hydrophobic power of non-aqueous solvents.

In the other test, SVP and its mutants were maintained in the presence of 40% of the mentioned organic solvents at 30 °C with continuous shaking for 15 days (Fig. 4). These results displayed that although all mutants increase the remaining protease activity in 15th day of incubation in the organic solvent but N248G and Y23V mutants show more values in the remaining protease activity in 15th day of incubation in the organic solvent. Y23V variant not only increases the remaining protease activity about 12 and 34% in DMF and methanol but also increase it about 20 and 30% in isopropanol and n-propanol (Fig. 4). Although, D25P improve the remaining protease activity in 15th day of incubation in n-propanol about two times compared to SVP, but N248G and Y23V improve it about 2.3 and 2.6 times compared to SVP.

Some common methodologies to engineering the thermo-tolerant enzymes have developed from an extensive arrangement of protein engineering tests [63], and abundant of this effort is appropriated to tolerant in non-aqueous solvents. On the foundation of this knowledge, as well as interpretations of normal proteins that are tolerant in non-aqueous solvents, a set of universal strategy guidelines for tailoring the organic solvent-tolerant enzymes has been suggested [64]. These guidelines were tested by rational mutagenesis of subtilisin E and α-lytic protease. The offered plan for stabilizing of α-lytic protease in the presence of an organic solvent was to exchange surface charges by exchanging charged amino acids with more hydrophobic ones [65]. Consequently, a mutated α-lytic protease taking two replacements was established to be more stable in 84% DMF at 30 °C than the wild-type enzyme under the equal situation or in an aqueous reaction at 60 °C. A L307V mutant of phenylalanine dehydrogenase which exhibited high function in non-aqueous solvent mixtures was also performed [66].

However, our results show a rational strategy for improving enzyme efficiency in the presence of organic solvents, in which the surface polar and charged residues substituted with hydrophobic ones. In addition, these results indicate that the N-terminal domain of TLPs not only responsible for enzyme stability in aqueous solvent, but also has a significant role in enzyme efficiency in the existence of organic solvents.

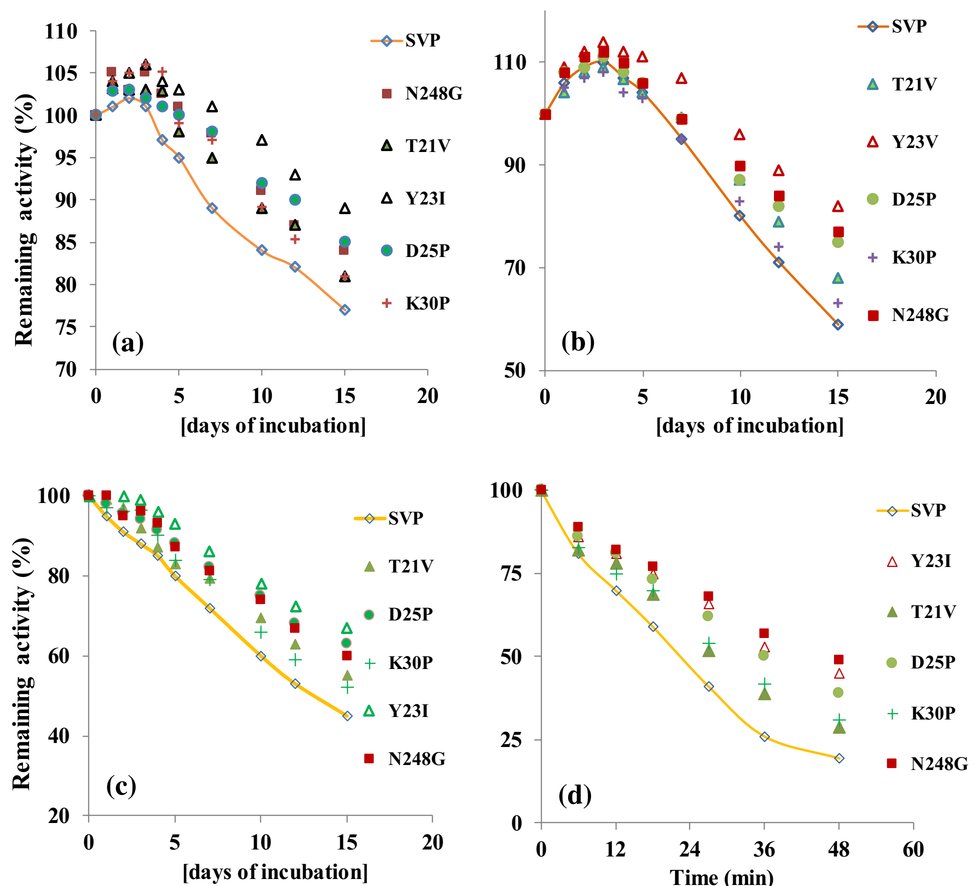

Fig. 4 The Residual protease activity of SVP [open diamond] and [T21V (filled diamond), Y23V (filled triangle), K30P [×], D25P (filled circle)] and N248G (open triangle)] in DMF (**a**), methanol (**b**), isopropanol (**c**) and propanol (**d**) with final concentration of 40% (V/V). The samples were incubated at 30 °C with 170 rpm for 15 days. In each day, samples were removed and their residual activities were determined as described in materials and methods section. Standards deviations were within 5% of experimental values

4 Conclusion

Our results indicated that enzyme efficiency improves with increasing the hydrophobic power of an organic solvent. Our methodical investigation on the protease efficiency in organic solvent, in polar and hydrophobic organic solvents results in the subsequent conclusions:

- The relative C_{50} of all mutants has more values in the hydrophobic organic solvents than polar organic solvents.
- kcat, kcat/*K*m parameters and $\Delta\Delta G^{\ddagger}$ values of all variants increase with increasing the hydrophobic power of organic solvents compared to SVP. The observed decrease in the inactivation rate in organic solvents compared to aqueous media might be due to the diminished auto-digestion, which itself is the consequence of the fall in the activity of the enzyme in organic solvents.
- Thermal stability results show that Y23V and N248G variants not only are the most effective mutant to improve enzyme activity but also they show the lowest k_i values between these mutations compared to SVP. These results also show that irreversible thermo-inactivation rate has straight relationship with the hydrophobic power of organic solvents.
- The results indicate that N248G and Y23V mutants show more values in the remaining protease activity in 15th day of incubation in organic solvent, and it has more value in hydrophobic organic solvents than polar ones. Finally, these results suggested a rational approach to develop protease function in organic solvent media.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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