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Screening, Characterization, and Cloning of a Solvent-Tolerant Protease from *Serratia marcescens* MH6

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A solvent-tolerant bacterium strain, MH6, was isolated by hydrophilic organic solvent DMSO enrichment in the medium and identified as *Serratia marcescens*. The extracellular protease with novel organic-solvent-stable properties from strain MH6 was purified and characterized. The molecular mass of the purified protease was estimated to be 52 kDa on SDS–PAGE. The open reading frame (ORF) of the MH6 protease encoded 504 amino acids with 471 amino acid residues in the mature protease. Based on the inhibitory effects of EDTA and 1,10-phenathroline, the MH6 protease was characterized as a metalloproteinase. The enzyme activity was increased in the presence of Ni^{2+}, Mg^{2+}, and Ca^{2+}. The protease could also be activated by the nonionic surfactants Tween 80 (1.0%) and Triton X-100 (1.0%). The protease showed remarkable solvent stability in the presence of 50% (v/v) solutions of long-chain alkanes and long-chain alcohols. It was also fairly stable in the presence of 25% solutions of hydrophilic organic solvents. Owing to its high stability in solvents and surfactants, the MH6 protease is an ideal candidate for applications in organic catalysis and other related fields.

Keywords: Organic solvent tolerance, metalloproteinase, screening, nonaqueous enzymology, *Serratia marcescens*

The application of enzymes to reactions performed under nonaqueous conditions has become common practice in biocatalytic processes [20, 21]. Organic solutions are ideal for synthesis reactions since the solubility of polar substrates increases in such solutions. Moreover, reversal of the thermodynamic equilibrium favors synthesis over hydrolysis, suppresses water-dependent side reactions, and decreases microbial contamination. Such reactions are also characterized by high selectivity, specificity, and catalytic rates [8, 10]. To suit the conditions that prevail in practical production, enzymes must be stable in the presence of organic solvents. In recent years, new proteases that are stable in organic solvents have been discovered [6, 7, 12, 16]. These proteases have been successfully applied to the synthesis of several peptides such as the aspartame precursor [29, 31], kyotorphin precursor [25], and Bz-Arg-Gly-Asp (-OMe)-OH (RGD) [28].

Some soil microorganisms from the contaminated area can adapt to the presence of various organic solvents and undergo rapid adjustment to survive changes in the external solvent concentration [34]. This property of tolerating organic solvents makes these bacteria better candidates for exploiting naturally solvent-stable enzymes. Solvent-tolerant bacteria have been isolated from the soil, coastal sediment, deep sea, *etc*. However, most of the reported strains belong to genera such as *Pseudomonas*, *Bacillus*, *Arthrobacter*, and *Rhodococcus* [7, 9, 12, 13]. The demand for potentially useful proteases with specific properties continues to stimulate the search for new sources of organic-solvent-tolerant proteases. Moreover, the discovery of new bacteria or enzymes may open up new areas of basic research in the fields of nonaqueous enzymology and applications.

This report describes a novel solvent-tolerant characteristic of protease from a hydrophilic organic-solvent-tolerant microorganism, *Serratia marcescens* MH6. The MH6 protease was purified and characterized. The gene encoding the organic-solvent-tolerant protease was also cloned. The results expended the source of solvent-stable protease for nonaqueous biocatalysis.

Materials and Methods

Materials

DEAE-Sepharose Fast Flow was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Casein was purchased from Sigma (St. Louis, MO, U.S.A.). Tryptone and yeast extract were products of Oxoid (Basingstoke, Hampshire, U.K.). LA-*Taq* DNA polymerase and the PMD-18T vector were purchased from Takara (Dalian, China).

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DH5α (Clontech, Saint-Germain-en-Laye, France) was used as the *E. coli* host. All other chemicals used were of analytical grade.

Methods

Screening of Organic-Solvent-Tolerant Microorganisms

Samples were collected from oil- or chemical-contaminated soils from various parts of China. Organic-solvent-tolerant bacteria were screened from soil samples by dimethylsulfoxide (DMSO) enrichment in modified Luria–Bertani (MLB) medium containing the following (in g/l): tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; and $MgSO_4$, 0.5. The MLB plate medium had the same composition but also contained agar (1.8%). Organic solvents were added to the medium to a concentration of 10%~20% (v/v). The bacteria were cultivated in 25 ml of MLB medium in 250-ml Erlenmeyer flasks maintained at 30°C. Incubation with agitation was carried out at 180 rpm for 24 h. The cultures were then acclimated by repeated transfer to fresh MLB medium containing the organic solvents. Samples from repeated batch cultures were diluted and spread on MLB plates, and the growing colonies were further purified by repeated streaking.

Isolation of Strains That Produce a Solvent-Tolerant Protease

Isolated colonies on the plates containing the MLB medium were then streaked onto another selective skim milk agar (SMA) plate for crude estimation of the proteolytic activity. These plates were incubated at 37°C for 24 h. The SMA plate contained the following (in g/l): tryptone, 5.0; yeast extract, 8.0; skimmed milk powder, 10.0; and bacteriological agar, 18.0. The microbes that showed high ratios of the clear zone diameter to the colony diameter at an early stage were selected as potential protease producers. The organic solvent stability of the protease was determined as follows. The strains were cultured in MLB liquid medium at 37°C and 200 rpm. After incubation for 24–48 h, the crude protease was recovered by centrifugation at 10,000 ×*g* and 4°C for 15 min. Different organic solvents were added to a final concentration of 50% (v/v) to 1 ml of crude protease, and the reaction mixture was incubated at 30°C and 180 rpm for 24 h. The residual protease activity was then assayed. Crude enzyme without any organic solvent was used as the control. The strain that produced a protease with wide organic solvent stability was selected. The isolated strains were then identified by 16S rDNA sequence homology search, biochemical tests, and Biolog Automated Microbiology Identification.

Protease Production

For bacterial growth, the inoculums were prepared by inoculating a loopful of fresh cultured strain from the plate into 20 ml of LB medium followed by incubation at 37°C and 180 rpm. One ml of the overnight culture broth was used to inoculate 35 ml of the optimized medium in a 250-ml Erlenmeyer flask containing the following (in g/l): tryptone, 10.0; yeast extract, 10.0; $(NH_4)_2SO_4$, 1.5; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2{\cdot}2H_2O$, 1.0; NaCl, 1.0; and glycerol, 5.0 ml. The pH of the medium was adjusted to 7.5 with NaOH. The cells were incubated at 30°C with constant shaking on an orbital shaker at 200 rpm. After 28–30 h of growth, the cells were harvested by centrifugation at 10,000 ×*g* for 15 min at 4°C. The supernatant thus obtained was used as the crude protease.

Determination of the Protease Activity and Protein Concentration

Protease activity was determined by casein digestion using a modified method described by Shimogaki *et al*. [26]. The reaction mixture consisted of 200 μl of diluted enzyme solution (enzyme:buffer, 1:50) and 200 μl of 0.05 M Tris-HCl buffer (pH 8.0) containing 2% (w/v) casein, which had been preincubated at 40°C for 10 min. The reaction mixture was then incubated at 40°C for 10 min, and the reaction was terminated by adding 500 μl of a trichloroacetic acid (TCA) mixture (containing 0.11 M TCA, 0.22 M sodium acetate, and 0.33 M acetic acid). The mixture was further incubated at 40°C for 10 min, followed by centrifugation at 12,000 ×*g* for 20 min. The supernatant was harvested, and the absorbance was measured at 280 nm. The "blank" was prepared by the same procedure with TCA being added at zero time. One unit (U) of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per minute under the conditions described above. The protein concentration was determined by the Bradford method [2].

Protease Purification

The MH6 protease was purified in two steps. All procedures were performed at 4°C. After incubating *S. marcescens* strain MH6 in the optimized medium for 28 h, the culture was centrifuged at 10,000 ×*g* for 15 min to remove the cells. The culture supernatant was fractionated by adding ammonium sulfate to 50%–60% saturation. The protein precipitates were collected by centrifugation at 9,000 ×*g* for 20 min and dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enzyme solution was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) for 24 h with three buffer changes. The dialyzates were passed through an ion-exchange DEAE-Sepharose Fast Flow (DEAE-Sepharose FF) column that had been pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The proteins were eluted by a linear gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 1 ml/min. The fractions with protease activity were pooled and concentrated by centrifugation using a 10-kDa molecular mass cut-off Centricon device (Millipore, U.S.A.). The purity and molecular mass of the protease were analyzed by SDS–PAGE. The purified protease was used for further studies.

Effects of pH and Temperature on the Activity and Stability of the Protease

To determine the effect of pH on the activity of the MH6 protease, the purified protease was incubated for 30 min at 4°C in the presence of different buffers. Casein was used as the substrate, and the protease activity was measured by the assay method described above. To determine the optimum temperature for enzyme activity, the enzyme was incubated with casein substrate in 50 mM Tris-HCI (pH 8.0) at different temperatures. Samples were removed at fixed time intervals and allowed to cool in an ice bath. The residual protease activity was compared with the optimum activity and expressed as the relative activity.

Effects of Metal Ions and Inhibitors on Protease Activity

The effects of different metal ions, including Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} (in the form of chlorides), was tested by preincubating the purified protease with 1 mM and 5 mM solutions of these ions at 4°C for 30 min. The residual activity was measured under standard assay conditions. The effects of inhibitors on protease activity was examined by preincubating the protease for 1 h at 4°C with phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and 1,10-phenathroline. The final concentration of the inhibitors in the preincubation mixture was 5 mM. The residual activity was calculated with reference to the protease activity in the absence of supplements. A similar assay was conducted to determine

the effects of different reagents [β-mercaptoethanol, dithiothreitol (DTT), and urea] and surfactants [sodium dodecyl sulfate (SDS), cetyldimethylethyl ammonium bromide (CTAB), Tween-80, and Triton X-100 all at 1% (v/v)] on protease activity.

Effects of Organic Solvents on Protease Stability

Various organic solvents with different log *P* values were chosen in the study of organic solvent stability. One ml of organic solvent was added to 1 ml or 3 ml of purified protease solutions, and the reaction mixtures were incubated at 30°C with shaking of 180 rpm in universal bottles. Aliquots were withdrawn after 48 h to determine the residual activity. In the case of water-immiscible solvents, it is important to carefully withdraw the samples from the aqueous phase. The stability was expressed as the residual proteolytic activity relative to the non-solvent-containing control (0%). Each experiment was performed in triplicates.

Protein Identification by Mass Spectrometry (MS) and Cloning of the MH6 Protease

The purified protease was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the Coomassie-stained band of the purified protease was excised from the gel and submitted to the National Center of Biomedical Analysis (NCBA, Beijing) for MS analysis. The trypsin-digested protein fragments of the protease were subjected to LC/MS/MS analysis, and the data thus generated were submitted to the Mascot program for possible identity matching [4].

The genomic DNA of *S. marcescens* strain MH6 was obtained by the standard phenol/chloroform precipitation method.

The sense primer 5'-GTGGCTTACGGGGAGGTTAT-3' and the antisense primer 5'-TCCGTTGCTGTGTTACACGA-3' were designed based on the amino acid sequence of the *S. marcescens* metalloproteinase, since the MS analysis of the digest fragments of the purified protease showed that it was identical to the reported sequence of a metalloproteinase from *S. marcescens* [3]. PCR amplification was carried out in a total volume of 50 µl containing 1 µl of genomic DNA, 0.5 µl of LA-*Taq* enzyme, 5 µl of 10× *Taq* buffer ($MgCl_2$), 0.5 µl of dNTP, and 2 µl of the above primers. The following procedure was used: initial denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A final extension was performed at 72°C for 10 min. The amplified PCR fragment was purified by gel extraction and cloned into the PMD-18T vector and then sequenced.

RESULTS

Isolation a Solvent-Tolerant Protease-Producing Strain

Soil samples were collected from oil- or chemical-contaminated soils to screen for solvent-tolerant bacteria. DMSO was initially added to the medium to enrich solvent-tolerant microbes. Only eight isolates were obtained on the basis of their DMSO tolerance, and of these, six formed a clear zone on skim milk agar. Strain MH6 was selected as a potential protease producer on the basis of its distinct morphologies to the reported solvent-tolerant bacteria and significant tolerance of the crude protease to various solvents. The morphological characteristics of strain MH6 were confirmed by transmission electron microscopy (TEM) (Fig. 1). The strain was aerobic, Gram-negative, motile, catalase-positive, and rod shaped (0.7–1 µm in width and 1–1.5 µm in length). Strain MH6 was identified as *Serratia marcescens* after combining the analyses of the Biolog Automated Microbiology Identification System (SIM=0.51, 16–24 h) and 16S rDNA sequences (NCBI GenBank Accession No. FJ853424). In particular, the highest homology (99%) was observed with *S. marcescens*.

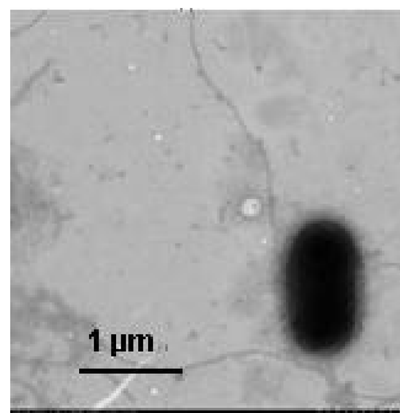

Fig. 1. Transmission electron micrograph of strain MH6 (Bar: 1 µm).

Purification of the Protease

The extracellular protease produced by *S. marcescens* MH6 was purified by gradient ammonium sulfate precipitation and ion-exchange chromatography and the results are summarized in Table 1. The fermentation supernatant was fractionated by adding ammonium sulfate to 50%–60% saturation with 69.1% yield. The precipitate was dissolved in Tris-HCl buffer (pH 8.0), and the dialyzate was loaded onto an ion-exchange DEAE-Sepharose Fast Flow column. The fraction with protease activity was collected and concentrated. Fig. 2 shows the results of the SDS–PAGE

Table 1. Purification of protease from *S. marcescens* MH6.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	74,672	10.53	7,091	100	1
$(NH_4)_2SO_4$ precipitation	51,535	5.04	10,221	69.10	1.44
DEAE–Sepharose Fast Flow	14,836	0.35	42,388	19.87	5.98

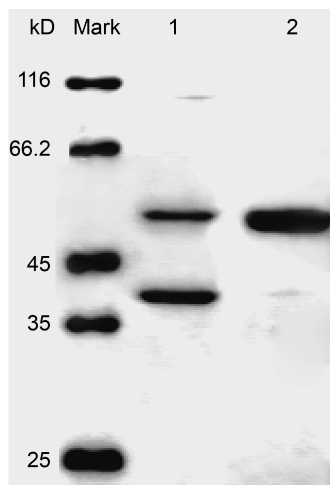

Fig. 2. SDS−PAGE of the protease purified from *S. marcescens* strain MH6.
Lane 1: culture supernatant, and Lane 2: purified protease. The molecular mass was estimated on the basis of standard proteins such as β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), and restriction endonuclease BSP981 (25 kDa).

analysis of the crude and purified proteases. The purified enzyme was homogenous with a molecular mass of 52 kDa. The enzyme was purified approximately 5.98-fold with an overall yield of 19.87%.

Effects of pH and Temperature

The purified protease was active over the pH range 7.0−8.5, with optimum activity at pH 8.0. However, the activity sharply decreased at pH values above 8.5 (Fig. 3). The

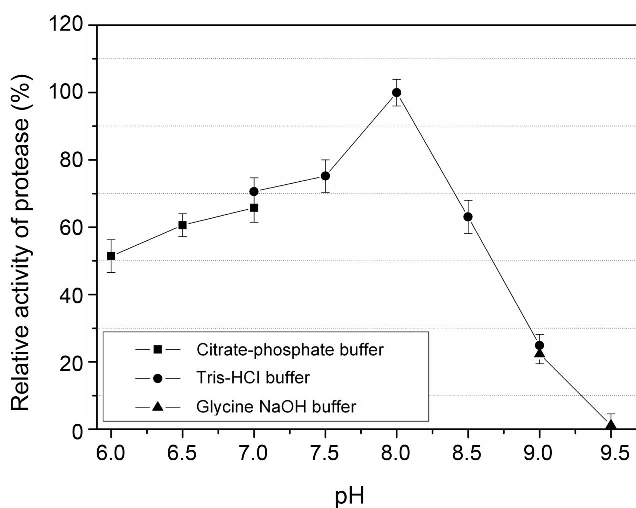

Fig. 3. Effect of pH on the activity of the purified MH6 protease.
Citrate-phosphate buffer was used in the pH range 4.0−7.0, Tris-HCl buffer was used in the pH range 7.0−9.0, and glycine-NaOH buffer was used in the pH range 9.0−10.0. Each value represents the mean of triplicate measurements.

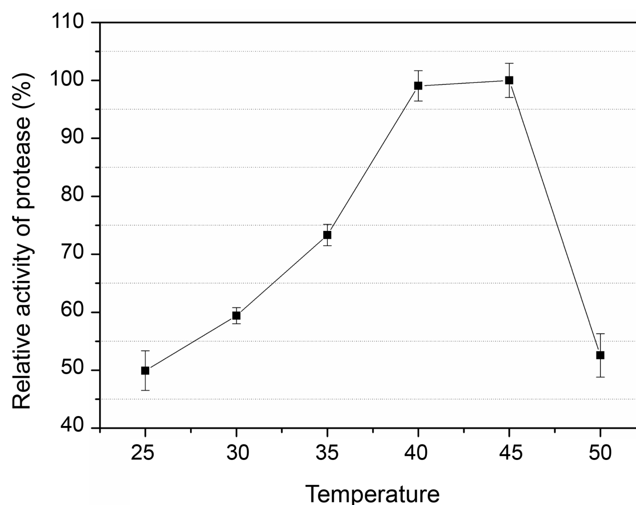

Fig. 4. Effect of temperature on the activity of the purified MH6 protease.
Each value represents the mean of triplicate measurements.

optimum temperature for protease activity was 45°C (Fig. 4). The enzyme retained 80% of its maximum activity after incubation for 60 min at 40°C but lost most of its activity after incubation for 30 min at 55°C (data not shown).

Effects of Metal Ions and Inhibitors

The effects of various metal ions and inhibitors on protease activity is summarized in Table 2. With the exception of Cu^{2+} and Zn^{2+}, metal ions at a concentration of 1 mM had a stimulatory effect on the protease activity. In the presence of 1 mM Ni^{2+}, the enzymatic activity increased by 16%. The purified protease was inhibited by metal chelators such as EDTA (5 mM) and 1,10-phenathroline (5 mM). This result suggests that the protease from strain MH6 belongs to the metalloproteinase family. Enzyme inhibition experiments showed that PMSF (5 mM), a serine protease inhibitor, had minor effects on the protease. Nonionic

Table 2. Effects of metal ions on the activity of the purified protease.

Metal ions	Relative activity (%)	
	1 mM	5 mM
None	100	100
Ni^{2+}	116	115
Mg^{2+}	110	90
Zn^{2+}	67	10
Ca^{2+}	113	72
Cu^{2+}	42	16
Mn^{2+}	107	44
Co^{2+}	101	62
Ba^{2+}	107	79

Each value represents the mean of triplicate measurements. The individual values did not vary from the mean by more than 10%.

Table 3. Effects of inhibitors and surfactants on the activity of the purified protease.

Reagent	Concentration	Relative activity
None	-	100
EDTA	5 mM	45
1,10-Phenathroline	5 mM	0
PMSF	5 mM	55
DTT	5 mM	32
Urea	5 mM	97
β-Mercaptoethanol	5 mM	17
SDS	1%	0
CTAB	1%	0
Tween 80	1%	103
Triton X-100	1%	104

Each value represents the mean of triplicate measurements. The individual values did not vary from the mean by more than 10%.

Table 4. Effects of organic solvents on the stability of the purified protease.

Organic solvents	Log *P*	Stability	
		25% (v/v)	50% (v/v)
None	-	1.00	1.00
n-Hexadecane	8.8	1.14	1.16
n-Tetradecane	7.6	1.12	1.20
n-Dodecane	6.6	1.08	1.03
n-Decane	5.6	1.08	1.00
n-Octane	4.5	1.07	1.22
n-Heptane	4.0	1.09	1.07
Cyclohexane	3.2	0.99	0.94
Acetic ether	0.68	1.13	1.37
DMSO	-1.0	1.00	0.50
DMF	-1.35	0.52	0.24
Isopropanol	0.05	0.67	0.10
n-Butanol	0.8	1.00	0.53
Isoamyl alcohol	1.3	1.10	1.00
n-Heptanol	2.4	1.23	1.12
1-Octanol	2.9	1.53	1.50
Decanol	4.0	1.57	1.53
n-Dodecanol	5.0	1.40	1.33

One ml of the various organic solvents was added to 1 ml or 3 ml of purified protease solutions, and these were incubated at 30°C and 180 rpm in universal bottles. Aliquots were withdrawn after 48 h to test the residual activity. Each experiment was carried out three times. The difference in the individual results of each set of experiments was less than 10%.

surfactants such as Tween 80 [1% (v/v)] and Triton X-100 slightly enhanced the protease activity, whereas 1% (v/v) solutions of the anionic surfactant SDS and cationic detergent CTAB strongly inhibited the protease activity. Sulfhydryl reagents such as dithiothreitol and β-mercaptoethanol significantly inhibited the protease. Urea at 5.0 mM concentration showed no effect on the protease (Table 3).

Stability of the MH6 Protease in Different Organic Solvents

Enzymes are generally inactivated in the system of organic solvents to the reaction solution. Table 4 shows the residual protease activities after incubation in various solvent systems for 48 h. The MH6 showed remarkable stability in the presence of hydrophobic solvents. Organic solvents such as most types of alkanes, long-chain alcohols, and acetic ether [50% (v/v)] enhanced the activity of the purified protease. The protease was stable in 25% solutions of DMSO and *n*-butanol, which are generally used to study the solvent stability of proteases [7, 15, 18]. However, protease was less stable in 50% DMSO, DMF, isopropanol, and *n*-butanol.

Determination of the Protein Sequence of the MH6 Protease

The internal peptides of the MH6 protein were identified by trypsin digestion and sequencing. The matching sequences included GNGIQINGK (fragment 1), FSSTNVAGDTGLSK (fragment 2), TGDTVYGFNSNTGR (fragment 3), and SFSDVGGLK (fragment 4). These sequences showed significant homology to the sequences of the metalloproteinase from *S. marcescens* SM6 [3]. Based on the nucleotide sequence of the metalloproteinase from *S. marcescens*, the gene encoding the organic-solvent-tolerant protease of *S. marcescens* MH6 was cloned. Homology search revealed that the MH6 protease gene had 98% identity with the metalloproteinase from *S. marcescens* SM6 (NCBI Accession No. X55521.1). The full-length open reading frame (ORF) of the MH6 protease consisted of 1,515 bp encoding 504 amino acid residues, and the mature protease contained 471 amino acid residues. BLAST-based analysis of the Swiss-Prot database also revealed that the amino acid sequence of the *S. marcescens* MH6 protease (between positions 18 and 504) showed the highest similarity to the zinc metalloproteinase from *S. marcescens* SM6. Four amino acids residues (Thr^{36}, Asp^{156}, Pro^{283}, and Ile^{365}) in the mature peptide from SM6 metalloproteinase were substituted with Ala, Gly, Leu, and Thr in the MH6 protease, respectively.

DISCUSSION

The main disadvantage of using organic solvents as media for biocatalysts is that the enzymes are easily inactivated or denatured. Therefore, different strategies have been employed to enhance enzyme activity and stability in non-conventional media [23]. However, it is more desirable to screen naturally evolved solvent-tolerant strains and enzymes instead of modifying the enzyme [15]. Hydrophobic organic solvents such as benzene, toluene, and cyclohexane are generally used in screening for solvent-tolerant bacteria [6,

7, 14]. Most solvent-tolerant bacteria belong to *Pseudomonas* sp. and *Bacillus* sp. Fang *et al*. [5] isolated 68 solvent-tolerant strains from marine mud samples by (10%) toluene enrichment in the medium. We previously used toluene and cyclohexane enrichment in the screening medium to isolate organic-solvent-stable protease producers from *Pseudomonas* [30] and *Bacillus* [12]. There are few reports on the isolation of organic-solvent-tolerant microorganisms using hydrophilic organic solvent enrichment. Organic solvents, especially hydrophilic organic solvents with low log *P* values (log $P<1$), are known to be extremely toxic to cells [34]. In this study, we modified the screening strategy to obtain distinct solvent-tolerant microorganisms. Using DMSO (log *P* −1.0) enrichment, only eight strains of organic-solvent-tolerant bacteria were isolated from more than 150 soil samples. These strains belonged to *Serratia marcescens*, *Stenotrophomonas maltophilia*, *Microbacterium*, and *Exiguobacterium*. This is a new genus in the reported solvent tolerance bacteria. The results indicated that these bacteria might have unique abilities to adapt to the environment in the presence of hydrophilic organic solvents. The influence of environmental factors is such that each bacterium has its own tolerance level for a particular organic solvent. For instance, the log *P* tolerance index values of strains *Pseudomonas*, *Arthrobacter* ST-1, and *Bacillus* SB1 are 2.5, 2.0, and 0.8, respectively [24].

S. marcescens produces many extracellular enzymes, including chitosanase, nuclease, lipase, hemolysin, serine, and thiol proteases, and metalloproteinase [1, 22, 32]. An organic-solvent-tolerant lipase from *S. marcescens* ECU1010 was reported to have a residual activity of 10% in DMSO and most water-immiscible organic solvents after 24 h of preincubation [35]. The chitosanase from *S. marcescens* TKU011 retained more than 50% of its activity in the presence of 25% (v/v) solutions of some organic solvents after incubation for 10 days at 4°C [33]. In this study, we examined the organic solvent tolerance of a metalloproteinase from *S. marcescens* MH6. The unique solvent tolerance of such microorganisms makes them good sources of organic-solvent-stable enzymes, especially their released extracellular enzymes.

The protease from *S. marcescens* MH6 was purified by a two-step purification procedure. The pH and temperature optima were 8.0 and 45°C. The protease was moderately inhibited by the sulfhydryl reagents β-mercaptoethanol and dithiothreitol, suggesting that there are disulfide bonds. Nonionic surfactants significantly enhanced the protease activity, whereas ionic surfactants such as SDS (anionic) and CTAB (cationic) were strong inhibitors of the MH6 protease, which suggested that there are a few ionic residues on the surface of the protease and these can easily be paired. Anionic and cationic surfactants are known to denature enzymes through interactions between the surfactants and the surface charge of the protein [11, 27]. The purified protease was inhibited by metal-chelating reagents such as EDTA and 1,10-phenanthroline, indicating that this protease is a metalloproteinase. The gene of the MH6 protease was cloned according to the analysis of its digested fragment sequence. BLAST analysis of the protease gene revealed that the amino acid sequence of the protease is also highly conserved and similar to that of the zinc metalloproteinase from *S. marcescens* with differences in only four amino acids. The MH6 protease was moderately activated by Ni^{2+}, Mg^{2+}, and Ca^{2+}. However, 1 mM and 5 mM Zn^{2+} significantly inhibited the MH6 protease activity, and the effect of Zn^{2+} was opposite to that of the Zn^{2+} protease from *S. marcescens* ATCC 25419. The activity of the protease from strain ATCC 25419 increases 8-fold in the presence of Zn^{2+} [22]. The differences in the metal-chelation properties might be closely related to the amino acid residuals near the active center of the proteases with high homology in the encoding gene from different strains of *S. marcescens.* The mechanism of differences in the effects of Zn^{2+} on the activities of the proteases from different strains of *S. marcescens* remains to be clarified.

In the present study, we demonstrated novel solvent-tolerant characteristics of a metalloproteinase from *S. marcescens* MH6. The protease was more stable in the presence of solvents with log *P* values higher than 1.3 (except cyclohexane) than in the absence of organic solvents. Moreover, the protease was quite stable in the presence of long-chain alcohols such as *n*-heptanol, 1-octanol, decanol, and *n*-dodecanol. It was also stable in the presence of 25% DMSO and butanol. Generally, solvents with log *P* values less than 4 are considered to be extremely toxic, since in such cases, essential water molecules present on the enzyme surface can be easily replaced with solvent molecules [15]. This phenomenon was also observed for a solvent-stable protease isolated from a solvent-tolerant strain of *P. aeruginosa* [7]. The gene encoding the solvent-tolerant protease from *S. marcescens* MH6 showed an at least four amino acid residues difference with the protease from strain SM6. Four amino acid residues of the protease from *S. marcescens* SM6 were polar amino acids (Asp^{156}, Thr^{36}), and nonpolar amino acids (Pro^{283}, Ile^{365}). The four mutations include three hydrophobic residues (Ala, Gly, and Leu) and one uncharged amino acid (Thr). The increased number of hydrophobic and uncharged amino acid residues on the MH6 protease might contribute to the organic solvent stability of the protease. Comparison of the protease from *P. aeruginosa* PST-01 with mutants indicated that the amino acid residues located at the surface of the protein molecule play an important role in the organic solvent stability of the enzyme [17]. Rahman *et al*. [19] also speculated that slight changes in the amino acid composition of the protease from *B. pumilus* 115b played an important role in the organic solvent stability of the protease. Further studies on this protease could provide an insight into the structure–

function relationships of its solvent tolerance and nonaqueous biocatalysis. The stability of the protease in various organic solvents makes it ideal for application to catalysis in organic solvent systems, particularly to biphasic catalysis.

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REFERENCES

1. Aiyappa, P. S. and J. O. Harris. 1976. The extracellular metalloprotease of *Serratia marcescens*: I. Purification and characterization. *Mol. Cell Biochem.* **13:** 95−100.
2. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248−254.
3. Braunagel, S. C. and M. J. Benedik. 1990. The metalloprotease gene of *Serratia marcescens* strain SM6. *Molec. Gen. Genet. MGG* **222:** 446−451.
4. David, N. P., J. C. P. Darryl, M. C. David, and S. C. John. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20:** 3551−3567.
5. Fang, Y. W., S. Liu, S. J. Wang, and M. S. Lv. 2009. Isolation and screening of a novel extracellular organic solvent-stable protease producer. *Biochem. Eng. J.* **43:** 212−215.
6. Geok, L. P., C. N. A. Razak, R. N. Z. Abd Rahman, M. Basri, and A. B. Salleh. 2003. Isolation and screening of an extracellular organic solvent-tolerant protease producer. *Biochem. Eng. J.* **13:** 73−77.
7. Gupta, A. and S. K. Khare. 2006. A protease stable in organic solvents from solvent tolerant strain of *Pseudomonas aeruginosa*. *Bioresource Technol.* **97:** 1788−1793.
8. Iyer, P. V. and L. Ananthanarayan. 2008. Enzyme stability and stabilization - Aqueous and non-aqueous environment. *Process Biochem.* **43:** 1019−1032.
9. Kato, C., A. Inoue, and K. Horikoshi. 1996. Isolating and characterizing deep-sea marine microorganisms. *Trends Biotechnol.* **14:** 6−12.
10. Klibanov, A. M. 2001. Improving enzymes by using them in organic solvents. *Nature* **409:** 241−246.
11. Lalonde, J., E. Witte, M. O'Connell, and L. Holliday. 1995. Protease stabilization by highly concentrated anionic surfactant mixtures. *J. Am. Oil Chem. Soc.* **72:** 53−59.
12. Li, S., B. F. He, Z. Z. Bai, and P. K. Ouyang. 2009. A novel organic solvent-stable alkaline protease from organic solvent-tolerant *Bacillus licheniformis* YP1A. *J. Molec. Catal. B Enz.* **56:** 85−88.
13. Na, K. S., A. Kuroda, N. Takiguchi, T. Ikeda, H. Ohtake, and J. Kato. 2005. Isolation and characterization of benzene tolerant *Rhodococcus opacus* strains. *J. Biosci. Bioeng*. **99:** 378−382.
14. Niehaus, F., C. Bertoldo, M. Kahler, and G. Antranikian. 1999. Extremophiles as a source of novel enzymes for industrial application. *Appl. Microbiol. Biotechnol.* **51:** 711−729.
15. Ogino, H. and H. Ishikawa. 2001. Enzymes which are stable in the presence of organic solvents. *J. Biosci. Bioeng.* **91:** 109−116.
16. Ogino, H., S. Nakagawa, K. Shinya, T. Muto, N. Fujimura, M. Yasuda, and H. Ishikawa. 2000. Purification and characterization of organic solvent-stable lipase from organic solvent-tolerant *Pseudomonas aeruginosa* LST-03. *J. Biosci. Bioeng.* **89:** 451−457.
17. Ogino, H., T. Uchiho, N. Doukyu, M. Yasuda, K. Ishimi, and H. Ishikawa. 2007. Effect of exchange of amino acid residues of the surface region of the PST-01 protease on its organic solvent-stability. *Biochem. Biophys. Res. Commun.* **358:** 1028−1103.
18. Rahman, R. N. Z. R. A., L. P. Geok, M. Basri, and A. B. Salleh. 2006. An organic solvent-stable alkaline protease from *Pseudomonas aeruginosa* strain K: Enzyme purification and characterization. *Enz. Microb. Technol.* **39:** 1484−1491.
19. Rahman, R. N. Z. R. A., S. Mahamad, A. B. Salleh, and M. Basri. 2007. A new organic solvent tolerant protease from *Bacillus pumilus* 115b. *J. Industr. Microbiol. Biotechnol.* **34:** 509−517.
20. Rai, S. K. and A. K. Mukherjee. 2009. Ecological significance and some biotechnological application of an organic solvent stable alkaline serine protease from *Bacillus subtilis* strain DM-04. *Bioresource Technol.* **100:** 2642−2645.
21. Rolland, V. and R. Lazaro. 2001. *Synthetic Applications of Enzymes in Nonaqueous Media*, pp. 357−371.
22. Romero, F. J., L. A. Garcia, J. A. Salas, M. Diaz, and L. M. Quiros. 2001. Production, purification and partial characterization of two extracellular proteases from *Serratia marcescens* grown in whey. *Process Biochem.* **36:** 507−515.
23. Roy, I., S. Sharma, and M. N. Gupta. 2004. *Smart Biocatalysts: Design and Applications*, pp. 251−310.
24. Sardessai, Y. and S. Bhosle. 2002. Tolerance of bacteria to organic solvents. *Res. Microbiol.* **153:** 263−268.
25. Sareen, R., U. T. Bornscheuer, and P. Mishra. 2004. Synthesis of kyotorphin precursor by an organic solvent-stable protease from *Bacillus licheniformis* RSP-09-37. *J. Molec. Catal. B Enz.* **32:** 1−5.
26. Shimogaki, H., K. Takeuchi, T. Nishino, M. Ohdera, T. Kudo, K. Ohba, M. Iwama, and M. Irie. 1991. Purification and properties of a novel surface active agent and alkaline resistant protease from *Bacillus* sp. Y. *Agric. Biol. Chem*. **55:** 2251−2258.
27. Shome, A., S. Roy, and P. K. Das. 2007. Nonionic surfactants: A key to enhance the enzyme activity at cationic reverse micellar interface. *Langmuir* **23:** 4130−4136.
28. So, J. E., J. S. Shin, and B. G. Kim. 2000. Protease-catalyzed tripeptide (RGD) synthesis. *Enz. Microb. Technol.* **26:** 108−114.
29. Tang, X. Y., B. Wu, H. J. Ying, and B. F. He. Biochemical properties and potential applications of a solvent-stable protease from the high-yield protease producer *Pseudomonas aeruginosa* PT121. *Appl. Biochem. Biotechnol.* **160:** 1017−1031.
30. Tang, X. Y., Y. Pan, S. Li, and B. F. He. 2008. Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. *Bioresource Technol.* **99:** 7388−7392.

31. Tsuchiyama, S., N. Doukyu, M. Yasuda, K. Ishimi, and H. Ogino. 2007. Peptide synthesis of aspartame precursor using organic-solvent-stable PST-01 protease in monophasic aqueous-organic solvent systems. *Biotechnol. Progr.* **23:** 820–823.
32. Ustariz, F. J., A. Laca, L. A. Garcia, and M. Diaz. 2008. Fermentation conditions increasing protease production by *Serratia marcescens* in fresh whey. *Revista Tecnica De La Facultad De Ingenieria Universidad Del Zulia* **31:** 79–89.
33. Wang, S. L., J. H. Peng, T. W. Liang, and K. C. Liu. 2008. Purification and characterization of a chitosanase from *Serratia marcescens* TKU011. *Carbohydr. Res.* **343:** 1316–1323.
34. Yogita, N. and S. B. Sardessai. 2004. Industrial potential of organic solvent tolerant bacteria. *Biotechnol. Progress* **20:** 655–660.
35. Zhao, L. L., J. H. Xu, J. Zhao, J. Pan, and Z. L. Wang. 2008. Biochemical properties and potential applications of an organic solvent-tolerant lipase isolated from *Serratia marcescens* ECU1010. *Process Biochem.* **43:** 626–633.