

微生物学报 *Acta Microbiologica Sinica*
55 (3) :311 – 320; 4 March 2015
ISSN 0001 – 6209; CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn
doi: 10.13343/j.cnki.wsxb.20140232

Characterization of solvent, detergent and oxidizing agent stable protease from isolated Antarctic marine *Streptomyces* sp. XE-1

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Abstract: [Objective] A polar marine actinobacterium (XE-1) was selected and used to produce a protease with special characteristics. [Methods] The XE-1 was identified as *Streptomyces* based on morphological, biochemical and molecular characterizations (16S rRNA gene sequence analysis). The protease was purified by 3 purification steps, including ethanol precipitation, ion exchange and gel chromatography. Its apparent molecular mass was estimated by SDS-PAGE. [Results] A solvent, detergent and oxidizing agent stable alkaline serine protease (with a low weight molecular, 14 kDa by SDS-PAGE), secreted by strain XE-1, was purified and characterized. The protease was stable in the pH range between 5 and 10, with optimal pH 8.2 and optimal temperature 55°C. K_m and V_{max} towards casein activity were 1.9 mg/mL and 973 U/mL, respectively. The protease was more active and stable in various hydrophilic organic solvents (such as dimethylformamid and toluene). Moreover, it was also active and stable in bleaching agents (such as hydrogen peroxide); and stable in denaturant agents (such as urea and guanidine hydrochloride) at the concentration from 0.2 mol/L to 4 mol/L, which were the new characteristics. [Conclusion] These biochemical characteristics suggest this enzyme has the potential value in numerous industrial applications.

Keywords: polar marine actinobacterium, low-molecular weight protease, solvent tolerant, detergent and oxidizing agent

CLC number: Q814 **Article ID:** 0001-6209 (2015) 03-0311-10

Proteases are the most important industrial enzymes with respect to their application in detergents, leather industry, food industry, pharmaceutical industry and others^[1]. The rate of protease growth in the number of patents issued to important industrial enzyme is 72% of the total industrial enzymes between 2010 and 2013^[2]. Protease can be produced from animals, bacteria,

fungi, and plants; microbial proteases are the most common enzymes among those^[1,3]. Recently, new bioresources from such marine environments especially the polar resources are explored regarding their potential to harbor new protease producers^[4-6]. In addition, the actinobacteria comprise significant components of the microbial population in most soil samples and have the

Supported by the Fundamental Research Funds for the Central Universities (JKZD2013017)

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Received: 5 May 2014 / Revised: 8 September 2014

characteristics of high stability and unusual substrate specificity, so the polar marine actinobacteria have recently emerged as rich sources for the isolation of industrial enzymes^[7].

Most of the proteases were secreted into the media by actinobacteria that differed from each another in their physical and biochemical properties, which had been characterized^[8-9]. Some had been reported about extracellular protease isolated from *Streptomyces lividans*, *Streptomyces griseus*^[10], *Streptomyces fradiae*, *Streptomyces albidoflavus*^[11], *Streptomyces pseudogrisiolus*^[9], *Streptomyces nogalator*^[12] and so on, while, as per our knowledge, protease from *Streptomyces griseorubens* has not been reported before.

Until recently, the molecular weight of extracellular protease produced by actinobacteria were general between 20 kDa and 70 kDa^[2], but it was rarely recovered a low molecular weight protease (below 20 kDa) from actinobacteria. Due to industrial demand of protease, most of effectors stable proteases were purified from bacteria, such as solvent stable, metal iron stable and surfactant stable proteases, but they were only stable at initial level with this effectors^[13]. In addition, most of enzymes were unstable in the present of denaturant agent and hydrogen peroxide. Based on our knowledge, only a protease from *Nocardiopsis kunsanensis* OM-6 could produce denaturant stable enzyme under the high concentration^[14] in many identified proteases.

In present study, we found a solvent, detergent and oxidizing agent stable protease with relative low molecular weight (14 kDa) from a polar marine actinobacterium. This study describes the production, purification and characterization of solvent, detergent and oxidizing agent stable protease. Moreover, the influence of different parameters on protease stability and activity is also determined in the following part.

1 Materials and Methods

1.1 Isolation, Identification and Cultivation

The marine actinobacterium, XE-1 was isolated

from soil sample which was collected from Antarctic Great Wall Station (62.22 °S, 58.96 °W), using the skim milk medium (W/V): yeast extract 1%; skim milk powder 2%. The phylogenetic position of XE-1 was established by amplifying the 16S rRNA gene and the sequence of the strain was analyzed by NCBI BLAST algorithm (<http://eblast.ncbi.nlm.nih.gov/>)^[15]. The DNA was amplified by PCR using the 16S rRNA gene primers: 27f: 5'-AGAGTTTGATCC TGGCTA-3', 1492r: 5'-GGTACCTGTTACGACTT-3'^[16]. Phylogenetic analysis was performed by using the neighbor-joining method^[17] by MEGA 5.1 program^[18]. Physiological and biochemical characteristics were determined according to the literature^[19]. The fermentation medium was with the following compositions: soybean 2%, starch 1%, baysalt 2.5%, Na₂HPO₄ 0.4%, CaCl₂ 0.2%, MgSO₄ 0.02%, K₂HPO₄ 0.03% and Na₂CO₃ 0.1%.

1.2 Protease Assay

Protease activity was measured by modified protocol as described by Sarath et al^[20]. With the casein as substrate, enzyme solution (400 μL) was added in a 400 μL aliquot of 1% (W/V) alkali soluble casein in 50 mmol/L phosphate buffer (pH 7.4). The reaction mixture was incubated at 40°C in a water bath for 20 min and reaction was stopped by adding 800 μL of 10% trichloroacetic acid, which kept at 25°C for 10 min and was centrifuged at 9000 × g for 2 min to remove the precipitate. Then, 1 mol/L Folin-Ciocalteu reagent (200 μL) was added to the mixture with the 200 μL supernatant and 1 ml of 0.4 mol/L sodium carbonate solution. This was further incubated for 20 min at 40°C for color development, which the optical density was at 680 nm. 1 Unit of enzyme activity was equal to 1 μg of tyrosine released per minute per mL at the above assay conditions.

1.3 Protein Assay

The protein content of individual fraction, obtained from different fractions of gel chromatograph, was monitored by measuring the extinction at 280 nm. Quantitative estimation of protein content was quantified by the method of Bradford using bovine-

serum albumin as a standard^[21].

1.4 Purification of Protease

The culture was harvested at $4000 \times g$ at 4°C and was fractionated into ethanol at a ratio of 1:2 (V/V) fermented extract/ethanol. Then, this solution was incubated for 30 min at 4°C and was subsequently centrifuged at $4000 \times g$ for 10 min at 4°C . The pellet obtained was resuspended in 50 mmol/L phosphate buffer, pH 7.4 and crude enzyme was concentrated by lyophilization. After that, the concentrated sample was separated by DEAE Sepharose 52 (Sigma) column. The bound enzyme was eluted by 50 mmol/L phosphate buffer, pH 7.4 at the flow rate of 1 mL/min, with a linear gradient of NaCl; 0 – 0.5 mmol/L. The active fraction was pooled, concentrated by lyophilization, resuspended in phosphate buffer, loaded onto a Superdex G-50 column equilibrated and eluted with phosphate buffer at a flow rate of 0.75 mL/min. The purified enzyme was stored at -20°C and was used to determine further biochemical characterization. The proteases from all purification steps were measured for enzyme activity and protein content, respectively.

1.5 Electrophoretic Analysis and Zymogram

The purity of protease and the molecular mass were judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli^[22] using a 15% separating gel. The protein was stained with silver^[23].

For zymogram analysis, SDS-PAGE was performed using 12% polyacrylamide containing 0.1% casein according to the method reported by Bernal et al^[24] with slight modification. After electrophoresis, the gel was rinsed with 2.5% Triton X-100 and incubated for 48 h at 25°C in 50 mmol/L Tris-HCl buffer (pH 7.4). Finally, the protease activity was visualized after Coomassie Brilliant Blue stain.

1.6 Effect of pH on Protease Activity and Stability

The optimum pH and stability of the purified enzyme were studied over a range of pH 3 – 12 with casein as substrate by measuring the activity at 40°C in

buffers with various pH values (sodium acetate, pH 3 – 6; sodium phosphate, pH 6 – 8; Tris-HCl, pH 8 – 9; glycine-NaOH, pH 9 – 11; $\text{Na}_2\text{HPO}_4\text{-NaOH}$, pH 11 – 12). For measurement of the pH stability, the enzyme was incubated at a given pH for 1 h at 25°C and the residual protease activity was determined under the above assay conditions (see 1.2).

1.7 Effect of Temperature on Protease Activity and Stability

The optimum temperature and stability of the purified enzyme were studied over a range of 25 – 80°C . When temperature stability was measured, the enzyme was incubated at a given temperature for 1 h at pH 7.4 and residual protease activity was determined under the above assay conditions (see 1.2).

1.8 Effect of Protease Inhibitors on Protease Activity

To assess the type of protease, the following inhibitors of protease activity were investigated: phenylmethylsulphonyl fluoride (PMSF), iodoacetate, metallo protease inhibitor diaminetetraacetic acid (EDTA), 2-mercaptoethanol, and dihiortitol (DTT). 400 μL of purified protease with each inhibitor (1 mmol/L/5 mmol/L) were preincubated at 25°C for 1 h, respectively. The residual protease activity was measured and the control was preincubated without any inhibitor as 100% under the above assay conditions (see 1.2).

1.9 Effect of Surfactants and Oxidizing Agent on Protease

The effect of surfactants on protease activity was investigated: Tween 20 and Tween 80, 0.4% – 1% (V/V); Triton X-100, 0.2% – 1% (V/V); sodium dodecyl sulphate (SDS), 0.2% – 1% (W/V); cetyl trimethyl ammonium bromide (CTAB), 0.1% /0.5% (W/V) and hydrogen peroxidethe (H_2O_2), 0.2% – 4%. The mixture was incubated at 25°C for 1 h, and the effects were assessed by considering the control (without effectors) as 100%.

1.10 Effect of Organic Solvents on Protease Activity

The effect of the following organic solvents on

protease activity was tested: acetone, dimethyl sulfoxide (DMSO), ethanol, ethyl acetate, isopropanol, dimethylformamid (DMF), toluene, and methanol at the 5% (V/V) and 25% (V/V), respectively. The mixture was incubated at 25°C for 1 h. A control was preincubated without organic solvent, which was considered as 100%. After incubation, the residual activity was measured under the above assay conditions (see 1.2).

1.11 Effect of Denaturant on Protease Activity and Stability

The effect of the following denaturants on the protease activity was investigated: urea (0 – 4 mol/L) and guanidine hydrochloride (0 – 4 mol/L). The mixture was incubated at 25°C for 1 h and the residual activity was assessed by considering the control (without denaturant) as 100%.

2 Results and Discussion

2.1 Isolation, Identification and Cultivation

The actinobacterium XE-1, was isolated from Antarctica and produced the largest clear zone on the skim medium, indicating it was a good protease producing strain. According to nucleotides homology and phylogenetic analysis (Figure 1), the sequence of strain XE-1 was highly homologous (99.93% pairwise similarity) with that of *Streptomyces griseorubens* NBRC 12780^T[25]. The GenBank accession number for strain XE-1 was KF938603. The characteristics of XE-1 were presented in Table 1. The results of this analysis showed that the strain XE-1 was Gram positive with filamentous structure and was different from *Streptomyces griseorubens* on biochemical characteristics. The strain XE-1 was used for further studies on extracellular protease production.

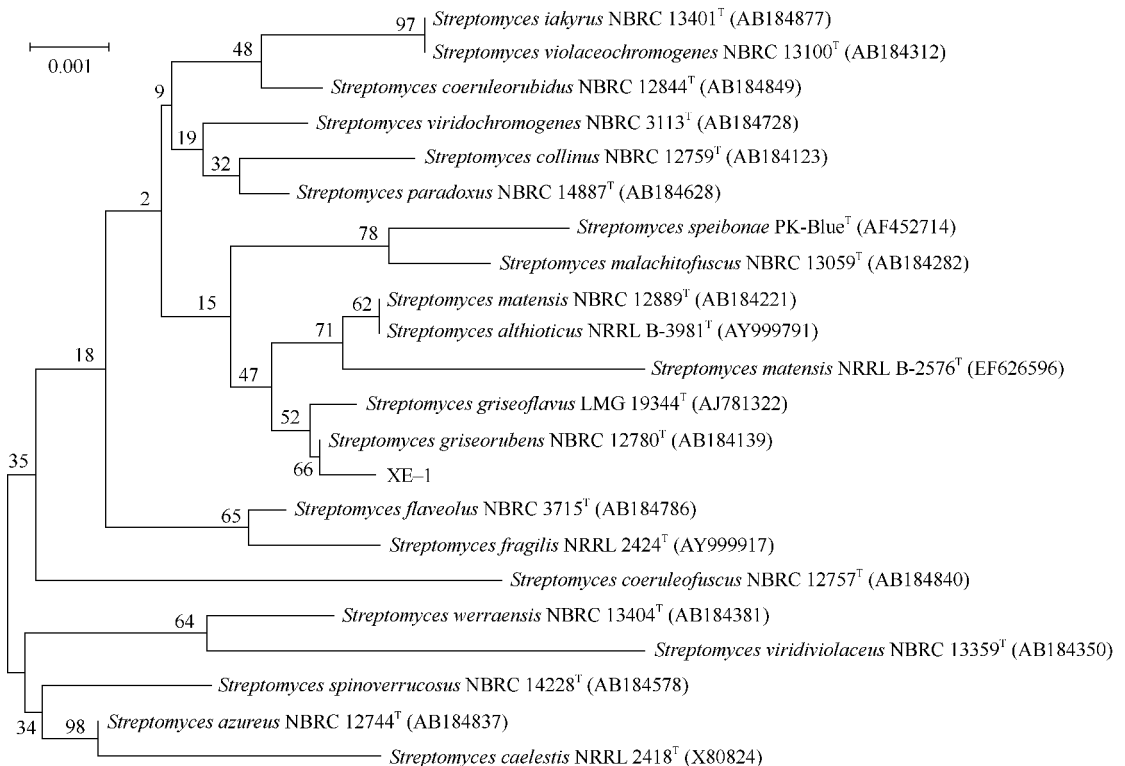


Figure 1. Phylogenetic tree base on neighbor joining analysis of 16S rRNA sequences showing the position of strain XE-1 and related taxa. Score bar represents 1 nucleotide substitution per 1000 nucleotides. Bootstrap values of 50 and above are shown. Accession numbers are shown in parentheses.

Table 1. Comparison of biochemical and physiological characteristics between *Streptomyces griseoruber* and strain XE-1

Characteristics	XE-1	<i>Streptomyces griseoruber</i> ^[26-27]
D-glucose	-	+
D-xylose	-	+
D-fructose	-	+
Rhamnose	-	+
D-mannitol	+	-
Cellulase	-	+
Lipase	+	-
Liquefaction of gelatin	-	+
Coagulation of milk	-	+

(-) was taken as not utilization and (+) was as utilization or production. The characteristics of I-inositol (+), sucrose (-), raffinose (-), protease (+), amylase (+), L-tyrosine (-), nitrate reductase (-), melanoid pigments (-) and H₂S production (-) were not different between the strain XE-1 and *Streptomyces griseoruber*.

Table 2. Summary of the purification of extracellular protease from strain XE-1

Purification step	Total protein/mg	Total activity/U _k	Specific activity/(U _k /mg)	Fold purification
Crude extract	5526	107688	19	1
Ethanol	270	91844	340	18
DEAE Sepharose 52	130	69016	531	30
Superdex G50	20	42110	2106	111

Then the protease purity and homogeneity were determined by SDS-PAGE. The purified protease was homogenous on SDS-PAGE. Combined with analysis, this result indicated that the enzyme was a monomer. Meanwhile, the apparent molecular weight of the purified protease was estimated to be 14 kDa based on SDS-PAGE (Figure 2-A). The value was lower than most other proteases from actinomycetes, which were ranged from 20 – 70 kDa and were commonly between 25 – 45 kDa^[11-12, 28-30]. Then the purified protease was subjected to substrate gel electrophoresis for confirmation of activity. Zymogram analysis released that only one enzyme band showed activity and indicated the molecular weight of activity band when stained with R250 Coomassie Brilliant Blue due to the hydrolysis of casein (Figure 2-B).

2.3 Optimal pH and Stability

The optimal pH of the purified enzyme was determined in the range of 3.5 – 12 pH using different buffer systems. The maximum activity of protease was observed in the range of 7.8 – 8.4 with the sodium

2.2 Purification and Molecular Mass

The protease was purified by ethanol precipitation, DEAE Sepharose 52 and gel filtration column chromatography. The overall results of the purification in each step were summarized in Table 2. The supernatant was resuspended with 50 mmol/L phosphate buffer after precipitated with using ethanol, which resulted in specific activity of 340 U/mg of protein. Following ethanol precipitation, the enzyme was purified over a DEAE Sepharose 52 column and the active fraction from the column using phosphate buffer without NaCl was concentrated and applied to a Superdex G-50 column. After the final purification, the protease was purified by 111 fold and the specific activity was 2106 U/mg.

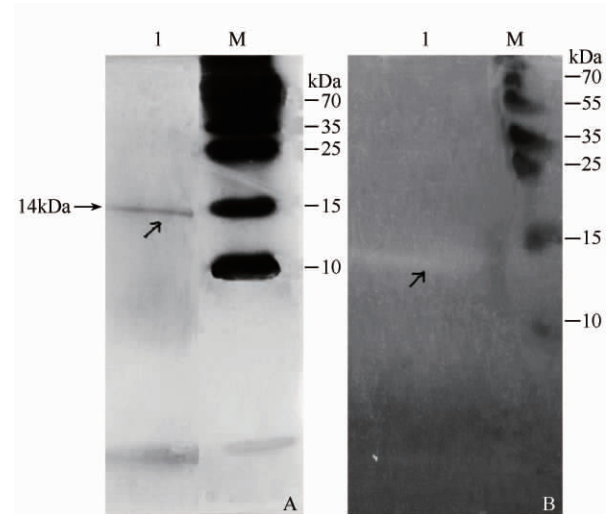


Figure 2. SDS-PAGE (A) and Zymography (B) of *S. XE-1* protease. Lane 1: purified protease. M: molecular weight markers (kDa). SDS-PAGE of the purified protease was under denaturing condition in a 12% gel containing 0.1% casein. Lane 1: purified protease. M: molecular weight markers (kDa).

phosphate buffer system (Figure 3-A). Meanwhile, different buffer systems also had an effect on protease activity. Enzyme activity in the sodium phosphate

buffer was higher than it in Tris-HCl buffer at the same pH 8.0, which was needed to attention in utilization of leather and other industries. The protease was fully stable over a wide pH range (from 5.0 to 9.0) for 1 h (Figure 3-B). In addition, the enzyme catalysis was only moderately stable, retaining 50% of the native activity at pH 4.5 and pH 11, suggesting it had a wide pH range to keep activity and to be used. In other words, the pH range was wider than that shown by several reports^[9,29,31].

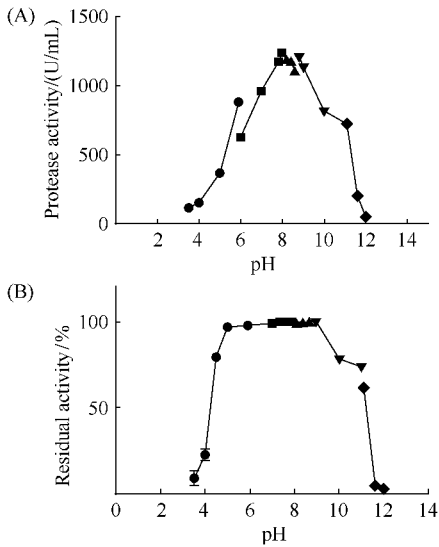


Figure 3. Effect of pH on protease activity (A) and stability (B). Each value represents the means of three experiments and the error bars indicate \pm SD.

2.4 Optimal Temperature and Stability

The optimal temperature was observed at 55°C, while the residual activity of enzyme was only 20% for 1 h at 80°C. The thermal inactivation experiment indicated that the enzyme was quite stable up to 45°C, while the protease stability linearly decreased with further increasing in temperature. The enzyme remained fully activity even after 60 min of incubation at 40°C but it lost most of activities at 60°C (Figure 4). The similar optimal temperature was in good agreement with other reports^[8,14,32]. We therefore concluded that the protease can catalytic the reaction immediately at a high temperature, but the value of activity was gradually decreased with the incubation time passed.

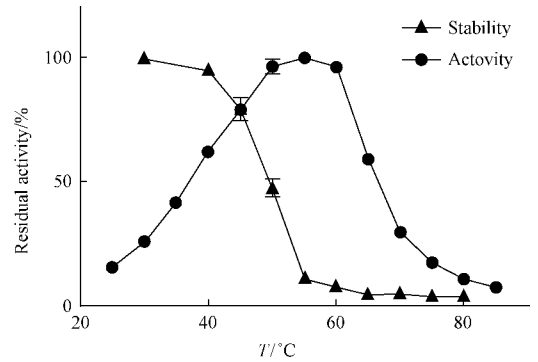


Figure 4. Effect of temperature on protease activity and stability. The maximum activity of the enzyme was taken as 100%. Data are presented as mean \pm SD calculated from three independent experiments.

2.5 Hydrolysis of Protein Substrate and Determination of K_m and V_{max}

Based on Lineweaver-Burk plot of the XE-1 protease, the kinetic parameters K_m and V_{max} , measured with casein as substrate, were 1.9 mg/mL and 973 U/mL, respectively. In this method, the production of tyrosine was used to estimate the velocity of the protease. It suggested the affinity to the substrate and reaction velocity, which performed well. Additionally, the casein was hydrolyzed efficiently by protease of the strain XE-1, BSA and gelatin not show hydrolysis phenomenon.

2.6 Effect of Protease Inhibitors and Metal Ions on Protease Activity

Protease inhibitors were employed to identify group at the active site of the enzyme (Table 3). Inhibitors like iodoacetate and mercaptoethanol did not affect enzyme activity, basically, but the purified enzyme was completely inhibited by PMSF, suggesting that the protease could be classified as serine protease. Other inhibitors like EDTA (5 mmol/L) and DTT (5 mmol/L) inhibited the protease activity by 23% and 11%, indicating that it contained metal ion near the active site.

2.7 Effect of Surfactants and Oxidizing Agent on Protease Activity

Proteolytic activity was not affected by Tween 80 at 0.4% to 1% concentration, but there were 28%, 80% and 27% reduction of protease activity in the

presence of 1% Triton X-100, 0.8% SDS and 0.5% CTAB, respectively (Figure 5-A). When 4% H₂O₂ existed (Figure 5-B), protease activity was slightly increased after 1 h of incubation at 25°C, suggesting that H-bond was not directly involved in protein stability and activity^[33]. This protease was used with several surfactants and basically maintained the full activity, except for SDS. Noticeable, the protease was also stable at the 100% of native activity and had the growth of activity under a high concentration of H₂O₂, even at 4%, which was rarely reported.

Table 3. Effect of various inhibitors on protease activity

Protease inhibitors	Concentration / (mmol/L)	Residual activity / %
PMSF	5	1.6 ± 0.7
iodoacetate	1	103 ± 1.0
	5	95 ± 0.2
	10	91 ± 0.8
EDTA	0.1	97 ± 1.9
	1	95 ± 3.7
	5	77 ± 3.8
mercaptoethanol	1	98 ± 3.2
	5	98 ± 2.3
	10	99 ± 1.6
DTT	1	95 ± 3.4
	5	89 ± 1.7

Data are presented as mean ± SD calculated from three independent experiments

2.8 Effect of Organic Solvents and Denaturant on Protease Activity

The purified protease shown highly stability in the presence of any organic solvent (5%, V/V) after 1 h incubation, which was prior to some protease^[34-36]. The activity of purified protease was stable at 50% of native activity, even if the concentration of acetone, DMSO, ethanol, ethyl acetate, isopropanol and methanol was high to 25% (V/V) (Figure 5-C). With the 25% toluene, the protease activity was stable at the native activity. Interesting, the protease activity was significant increased by DMF at the concentration of 25%, it has rarely been reported before^[29, 37-38]. With this feature, it was suggested that DMF was used as solvent of the protease, or the protease was used in reaction system with DMF.

The protease was subjected to denaturant at 0-4 mol/L urea and guanidine hydrochloride (Figure 5-D). In any tested concentration of that, protease activity was already maintained, even moderately increased. The enzyme activity was linearly increased by urea from 0.2 mol/L to 2 mol/L and was comparatively decreased at 4 mol/L urea, which was still higher than native activity. Then, the activity of protease was stable at 100% of initial activity, or moderately increased with the rise of concentration, which was similar with the report of Gohel et al^[14]. Interesting, XE-1 show the growth of activity compared with the OM-6^[14] under a high concentration, which was only resistant to denaturant at the same conditions.

3 Conclusion

In present study, a serine protease of small molecular weight was purified from an actinobacterium isolated from Antarctica. The protease was a low molecular weight (14 kDa) protein, which was rarely reported before and was not always from source with *Streptomyces griseorubens*. Though detailed advantage of low-molecular weight remains to be elucidated, we make a hypothesis that it may be contributed to the stability in structure and feature. So the structure of the protease is likely needed to be confirmed.

In addition, we attempted to understand the biochemical characteristics and kinetic properties. The protease was found to be highly resistant to oxidizing agent H₂O₂ (4%, V/V), organic solvents (5%, V/V) and some denaturant agents (4 mol/L) under the relatively high concentration, and had a broad pH and temperature range, a finding which is rather unique and restricted to only few proteins. The enzyme still retained protease activity even in 25% toluene, most of metal ions and some surfactant agents, and rarely lost full activity with other effectors. Interesting, the protease was highly stimulated by 25% DMF compared to other organic solvent (25%), which may be important for protease using. It means that the protease may be used in several fields, like peptide synthetic, detergent formulations and the leather industry.

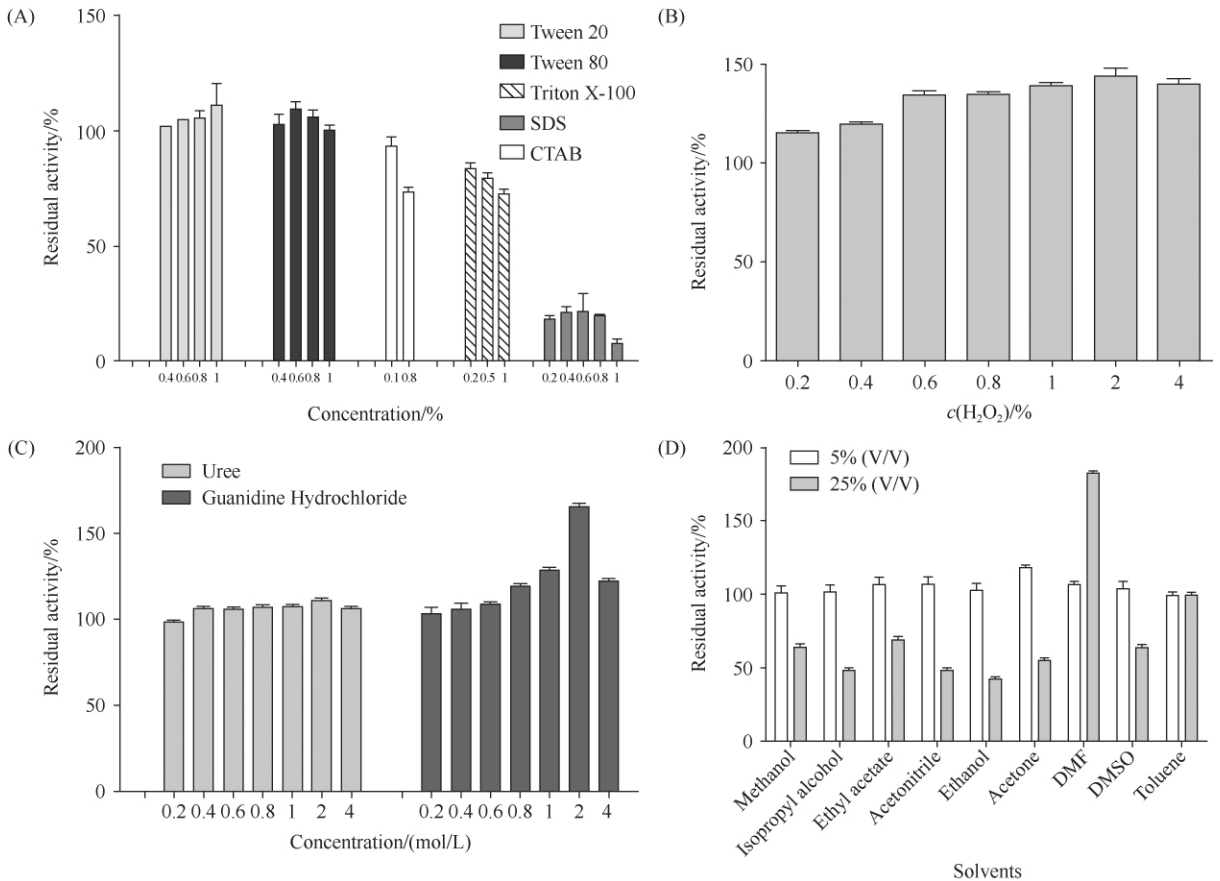


Figure 5. Effect of surfactant (A) : Tween 20 (W/W), Tween 80 (W/W), Triton X-400 (W/W), SDS (V/V) and CTAB (W/V); H₂O₂; (B) : from 0.2% to 4% concentration; denaturant agent (C) : from 0.2 to 4 mol/L concentration; organic solvent (D) : DMF, ethyl acetate, DMSO, methanol, acetone, acetonitrile, isopropyl alcohol, ethanol at 5% (V/V) and 25% (V/V) concentration. The activity protease without any agent was taken as 100%. Each value represents the means of three experiments and the error bars indicate \pm SD.

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南极海洋产耐有机溶剂变性剂氧化剂蛋白酶链霉菌菌株 XE-1 的初步鉴定及酶学性质

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摘要: 【目的】从南极海泥土壤中分离得到一株高产蛋白酶的放线菌(XE-1), 对所产蛋白酶进行纯化以得到具有特殊性质的酶产物。【方法】通过对 XE-1 的生理生化和 16S rRNA 基因序列分析, 初步将其确定为灰红链霉菌的变种。利用乙醇沉淀、离子交换、凝胶等方法纯化后, 利用冻干浓缩获得其胞外蛋白酶, 用 SDS-PAGE 测定分子量并进行性质表征。【结果】得到一种对高浓度的有机溶剂、变性剂、氧化剂耐受的, 表观分子量为 14 kDa 的碱性丝氨酸蛋白酶, 进一步分析, 其最适 pH 8.2, 温度为 55℃, 米氏常数 K_m 和 V_{max} 分别为 1.9 mg/mL 和 973 u/mL。此蛋白酶活性于 25% 的有机溶剂为 50% - 180% 的初始活性; 在不同浓度的氧化剂中为 110% - 140% 的初始活性, 在 0.2 mol/L 到 4 mol/L 的变性剂中表现为 100% - 150% 的初始活性。【结论】从南极海洋链霉菌 XE-1 中分离得到一种耐有机溶剂、变性剂、氧化剂, 大小约为 14 kDa 碱性蛋白酶, 其特殊的酶学性质可用于工业生产或药学等领域。

关键词: 极地海洋放线菌, 低分子量蛋白酶, 有机溶剂耐受, 变性剂和氧化剂

中图分类号: Q814 文章编号: 0001-6209 (2015) 03-0320-10

(本文责编: 张晓丽)

基金项目: 中央高校基础科研基金 (JKZD2013017)

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收稿日期: 2014-05-05; 修回日期: 2014-09-08