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Isolation, purification and characterization of extracellular protease produced by marine-derived endophytic fungus *Xylaria psidii* KT30

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PEER REVIEW

Peer reviewer

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Comments

This paper is an interesting study focusing on the extraction and biochemical characterization of an extracellular protease produced by the marine fungus *Xylaria psidii* KT30. The biochemical properties displayed by this molecule suggest its promising application in the field of biotechnology or medicine, obviously following further experimentation.

Details on Page 61

ABSTRACT

Objective: To isolate, purify and characterize extracellular protease produced by *Xylaria psidii* (*X. psidii*) KT30.

Methods: In the present study, the extracellular protease secreted by *X. psidii* KT30 was isolated and purified by using three steps of protein purification, then the purified protease was characterized by applying qualitative and quantitative enzymatic assays.

Results: Extracellular protease with molecular mass 71 kDa has been purified successfully by applying diethylaminoethanol-Sepharose followed by sephadex SG75 with its final specific protease activity of 0.091 IU/mg. Protease was the most active at temperature 60 °C and pH 7. The activity of enzyme was abolished mostly by phenylmethanesulfonyl fluoride, showing it is family of serine protease.

Conclusions: Extracellular serine protease produced by *X. psidii* KT30 with good biochemical properties displayed some promising results for its further application in field of biotechnology or medicine.

KEYWORDS

Serine protease, *Xylaria psidii* KT30, Bioactive molecule

1. Introduction

Fungal endophytes currently gain much attention in research in terms of their ability to produce diverse bioactive compounds that brings benefit in curing and preventing the emerging human diseases such as cancer and infectious diseases[1]. This group of microfungi is considered as one of factors that contribute in production and accumulation of many kinds of novel, unique and structurally broad spectrum of active metabolites in their hosts' habitats. This natural phenomenon occurs in fact that fungal endophytes tie a

symbiotic mutualism with host and their interaction mostly depends on virulence and defense equilibrium among fungi, host and pathogens[2].

Marine habitats are believed to be more rich in diversity compared to terrestrial environment that compose exclusively ecological interactions between microbotas with their microbiome and create one of the best and plenty sources for fungal endophytes-derived novel bioactive compounds to be exploited[3]. *Xylaria psidii* (*X. psidii*) KT30 is one of marine-derived fungal endophytes with great potency as source for bioactive compounds. Ethyl acetate extract

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of *X. psidii* KT30 broth culture showed antibacterial activity mostly against Gram-negative bacteria and some fish photogenic bacteria, and induced cytotoxicity against human bladder carcinoma cell line 5637[4]. Yet until now, the main active compound responsible for biological activity of *X. psidii* KT30 is still undergoing investigation. In this study, we try to reveal this active compound mainly focusing on proteases. The proteolytic enzymes have gained much attention to be explored due to their numerous biological activities such as antiviral[5], antibacterial[6], and anticancer activities[7].

Recently, almost all researches in novel bioactive compounds purified from fungal endophytes focused on non-protein secondary metabolites pathways-based new compounds isolations[8,9]; meanwhile, exploration of protein biomolecules as source for bioactive agents with high potency, especially from fungal endophytes, is still less attractive and this situation was sharp contrast to intensive research in investigating the new biomolecules with novel activity derived from macrofungi[10]. We do believe that biomolecules are more save compared to secondary metabolites, when applied as drugs or food preservatives in terms of their less toxicity effects and rapid metabolized by our body. In this study, we reported the isolation, purification and characterization of protease produced extracellularly by endophytic fungus *X. psidii* KT30.

2. Materials and methods

2.1. Materials

Culture of *X. psidii* KT30 was kindly obtained from Dr. Kustiariyah Tarman, Bogor Agricultural University, Indonesia. Diethylaminoethanol (DEAE)-cellulose was from Sigma Chemical Company, St. Louis, Missouri, USA. Sephadex SG-75 was from GE Healthcare Bio-Science, Uppsala, Sweden. Potato dextrose broth was from Himedia Laboratories Pvt. Ltd., India. Pierce bicinchoninic acid (BCA) protein assay kit and Pierce silver staining kit were from Thermo Scientific, USA. All other chemicals were purchased from Sigma Chemical Co.

2.2. Cultural condition

X. psidii KT30 was maintained on potato dextrose agar (PDA) at room temperature for 3–4 d at dark until the mycelia are mature enough to be recultured in liquid medium for extracellular proteases production. A mycelia, 0.5 mm×0.5 mm in length, was cultured in 300 mL of potato dextrose broth at 25 °C with 150 r/min for 10 d, then supernatant of the media was harvested for next experiment.

2.3. Purification of extracellular protease

Protein isolation was performed according to the protocol described by Wang *et al.* with slight modification[11]. Filtered-supernatant (crude extract) of 10-day *X. psidii* KT30 liquid culture was subjected to saturation using 90% solid ammonium sulfate, then resuspended in 25 mmol/L Tris-HCl buffer (pH 7.4). Before applied on DEAE-Sephadex for first step purification, resuspended pellet of crude extract was firstly dialyzed in dialysis tubing (23 mm×15 mm, Sigma, molecular mass cut-off 14 kDa) using the same buffer for 24 h at 4 °C with gently agitation, and the buffer was replaced twice during dialysis process. The dialyzed crude extract was then applied onto DEAE-Sephadex column pre-equilibrated with 25 mmol/

L Tris-HCl buffer (pH 7.4). Crude protein-containing DEAE-Sephadex column was separated using gradient concentration of NaCl (0–0.8 mol/L) with flow rate fixed at 1 mL/min. Fractions showing protease activity were pooled and then applied onto a sephadex SG-75 column against 25 mmol/L Tris-HCl buffer (pH 7.4) at a flow rate of 1 mL/min. Fractions with protease activity was freeze-dried and resuspended in the same buffer up to 25% of the original volume. The presence of protein was determined by BCA method at the wavelength of 540 nm with mixing 10 µL of sample with working solution.

2.4. Determination of protein concentration

Protein contents in each samples were measured using Pierce BCA protein assay kit as described in manual instruction. Bovine serum solution was used as standard proteins.

2.5. Directly extracellular protease assay on solid medium of culture

Explants of *X. psidii* KT30 mycelium (0.5mm×0.5mm in size) was sliced aseptically from original stock, then placed on 0.1% gelatin-containing PDA. The solid culture was incubated for 14 d in the dark at room temperature. At Day 7 and 14, the clear zone formation around *X. psidii* KT30 was monitored.

2.6. Screening of protease using plate assay

Plate assay described by Hasan *et al.*[12] and More *et al.*[13] was adapted with minor modification. Protease activity in each purification steps was monitored through clear zone formation on gelatin-added agarose. About 30 mL of 0.5% agarose medium containing 0.2% of gelatin in 25 mmol/L Tris-HCl buffer (pH 7.4) was placed on a Petri disc. Sample (50 µL) was loaded into well then incubated for 24 h at 37 °C for enzymatic reaction. The development of clear zone around the wells was detected by applying Coomassie Blue (0.25% w/v) in methanol: acetic acid: water 5:1:4 (v/v/v) for 15 min at room temperature, followed by destaining step to remove staining solution using destain solution (66 mL methanol: 20 mL acetic acid: 114 mL H₂O bidest) until the clear zone could be seen visually.

2.7. Protease activity assay

Activity of extracellular protease produced by *X. psidii* KT30 was measured using method developed by Cupp-Enyard with modification[14]. This method is based on the tyrosine formation during casein hydrolysis by protease and the tyrosine released was monitored at wavelength 540 nm. A total of 6 µL of test sample was firstly mixed with 6 µL phosphate buffer (pH 7.4, 25 mmol/L), then added with 6 µL of 1% (w/v) casein solution. The reaction mixture was incubated at 37 °C for 30 min. Following incubation, 12 µL of trichloroacetic acid was added to the mixture. After centrifugation at 12000 r/min for 1 min, reaction mixture was added with 143 µL of reagent A [a mixture of Na₂CO₃ solution and CuSO₄·5H₂O solution (5:1)], followed by adding 29 µL of Folin Ciocalteu reagent, and then stayed for 15 min before measured at 540 nm. A negative control was prepared by adding tricarboxylic acid cycle acid to the reaction mixture to prevent proteolytic reaction prior to addition of the substrate. One unit of protease activity is defined as 1 µmol of

tyrosine released during enzymatic reaction per mL reaction mixture per minute under the experimental conditions.

2.8. Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 12% polyacrylamide gel was used to determine purity of protein as described by Laemmli[15]. Protein on gel was visualized using silver staining reagents according to the manual instruction. The band of protein was compared to the standard molecular mass of marker to determine its molecular mass.

2.9. Gelatin zymography

The method described by Raser *et al.*[16] and Kleiner and Stevenson[17] was adapted. As mentioned, this method is very powerful not only to detect protease activity but also to predict molecular mass of the target protein. Firstly, the sample was run on 0.2% gelatin-containing gel electrophoresis. After separation of protein, the protein was reactivated by incubating the gel in 2.5% Triton X-100 for 40 min at 37 °C. The third step is staining the gel in 0.05% Coomassie Blue solution for 2 h. The final step is removing the excess Coomassie Blue using destaining solution until clear band appeared on gel which indicates the protease activity.

2.10. Determination of molecular mass of target protein

Molecular mass of protease was predicted from the protein band appeared on gel electrophoresis by interpolation from a linear logarithmic plot of relative molecular mass against the R_f value according to protocol explained by Martinez *et al.*[18]. The Precise Plus Protein Dual-Xtra standards from BioRad Laboratories Inc. was used as reference of protein.

2.11. Biochemical characterization

The effect of temperature, pH, and protease inhibitor on protease activity and its enzyme kinetic was evaluated following method described by Zhang *et al.* with modification[19]. The optimum temperature was determined by carrying out the assay at different temperatures ranging from 20 °C to 90 °C with 10 °C interval. Meanwhile, the optimal pH for protease activity was determined within the pH range 4-10 using the following buffers: 0.1 mol/L citrate buffer (pH 4-6), 0.1 mol/L Tris-HCl (pH 7-9) and 0.1 mol/L glycine-NaOH (pH 10-11). For inhibition effect assay, sample was treated with either protease inhibitors phenyl methyl sulfonyl fluoride (PMSF) or ethylenediaminetetraacetic acid (EDTA). The residual enzyme activity was measured by performing the assay as described above respectively. The value of K_m and V_{max} was determined based on the Lineweaver-Burk plot constructed by plotting the reciprocal of substrate concentration on the X-axis and reciprocal of the enzyme reaction velocity on the Y-axis by mixing sample with different concentration of casein ranging from 0.2%, 0.1%, 0.08%, 0.06%, 0.04%, and 0.02%.

3. Results

3.1. Assay of extracellular protease both directly on culture and fraction

The activity of fungal extracellular protease was confirmed by directly observation on solid medium. Proteolytic activity was shown by clear zone formation surrounding the active fungal growth as early as at Day 7 of incubation (Figure 1A). Plate assay showed protease activity particularly in resuspended-pellet of crude extract after ammonium sulfate precipitation, and also both in undialyzed and dialyzed sample (Figure 2).

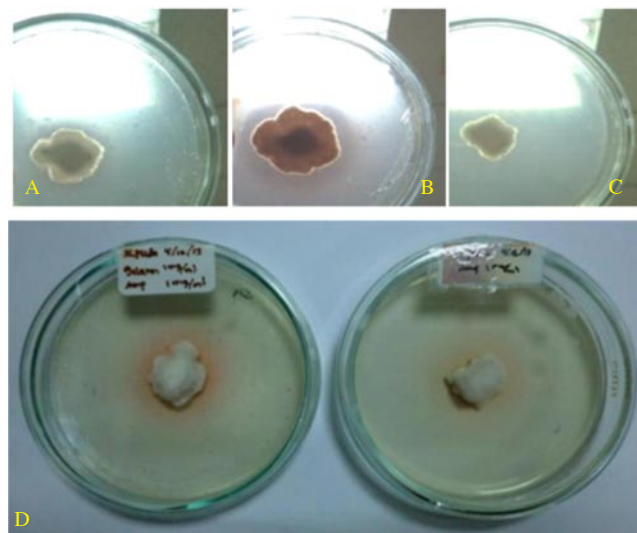


Figure 1. *X. psidii* KT30 on PDA medium.

A: At Day 7, clear zone formation of *X. psidii* KT30 on PDA medium containing 0.1% gelatin upon light exposure; B: At Day 14, clear zone formation of *X. psidii* KT30 on PDA medium containing 0.1% gelatin upon light exposure; C: At Day 7, *X. psidii* KT30 on PDA medium without gelatin upon light exposure; D: Culture of *X. psidii* KT30 with no direct exposure to light. Left is medium added with 0.1% gelatin and right is gelatin-free medium.

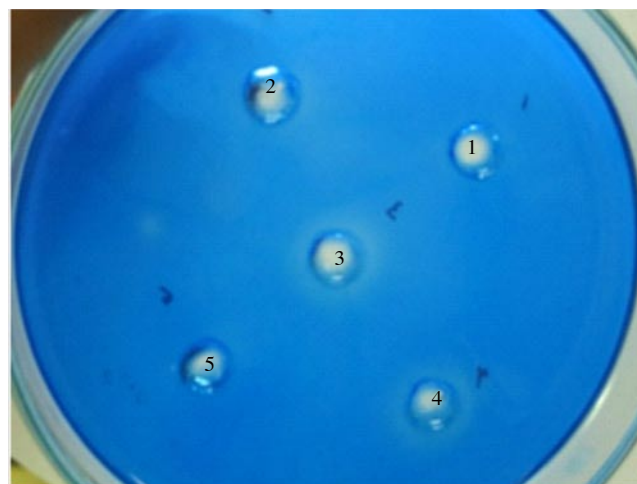


Figure 2. Results of plate assay.

1: Crude extract before ammonium sulfate precipitation; 2: Supernatant of crude extract after ammonium sulfate precipitation; 3: resuspended-pellet of crude extract after ammonium sulfate precipitation and before dialysis treatment; 4: resuspended-pellet of crude extract after dialysis treatment; 5: 25 mmol/L Tris-HCl with pH 7.

3.2. Purification of extracellular protease *X. psidii* KT30

Extracellular protease from *X. psidii* KT30 has been successfully purified from supernatant of 10-day medium culture by ammonium sulfate precipitation, DEAE-Sepharose column chromatography and sephadex SG75 column chromatography. Figure 3A exhibits two dominant peaks when anion exchanger column was eluted by gradient concentration of NaCl ranging from 0-0.8 mol/L and designed as Group A and Group B. Plate assay was applied on both groups to confirm the activity of protease in each fraction. As shown in Figure 3B only fractions on Group A showed dominant clear zone formation. Base on this result, the Group A was chosen to be applied in next step of purification. In the third step of purification by using sephadex-SG75, in total 40 fractions collected, only 7 fractions showed high peak when measured at wavelength 540 nm; and from those fractions only one fraction exhibited protease activity, SG75-F14, (Figure 4B and Figure 5A), which was detected by using gelatin zymography with molecular mass 43 kDa. Yet, this molecular mass was bigger when purified protein was separated on SDS-PAGE, showing single appearance, although the band appeared was very thin (Figure 5B). This protein band resulted from the final steps of purification was also detected on SDS-PAGE sample of Group A DEAE-column result. On the other hand, proteins with molecular mass above 71 kDa that appeared in second steps of purification disappeared after separated using sephadex-G75, indicating success of overall protein purification steps. Furthermore, the purity and protease specific activity of purified enzyme after all process of purification steps was 1.182 fold and 0.091 IU/mg respectively (Table 1).

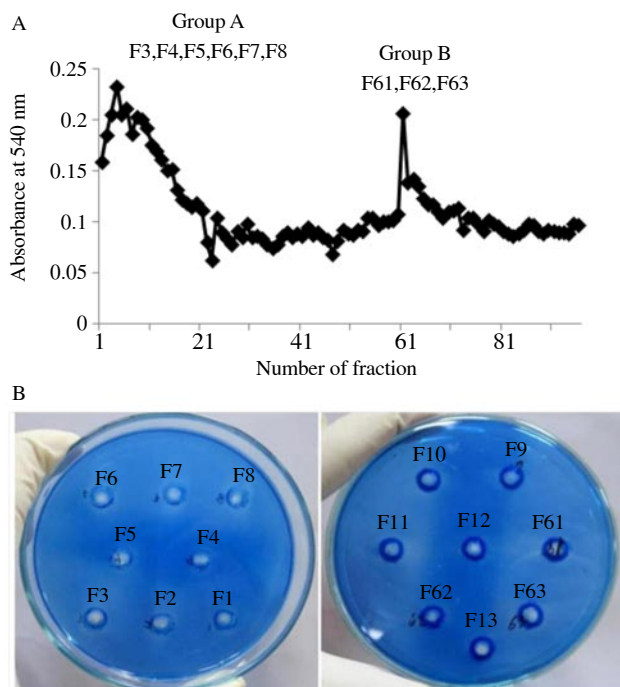


Figure 3. Chromatography profile and protease activity screening. A: DEAE-Sepharose chromatography profile of dialyzed-crude preparation; B: Protease activity screening on gelatin-containing agarose of samples resulted from anion exchange chromatography for purification.

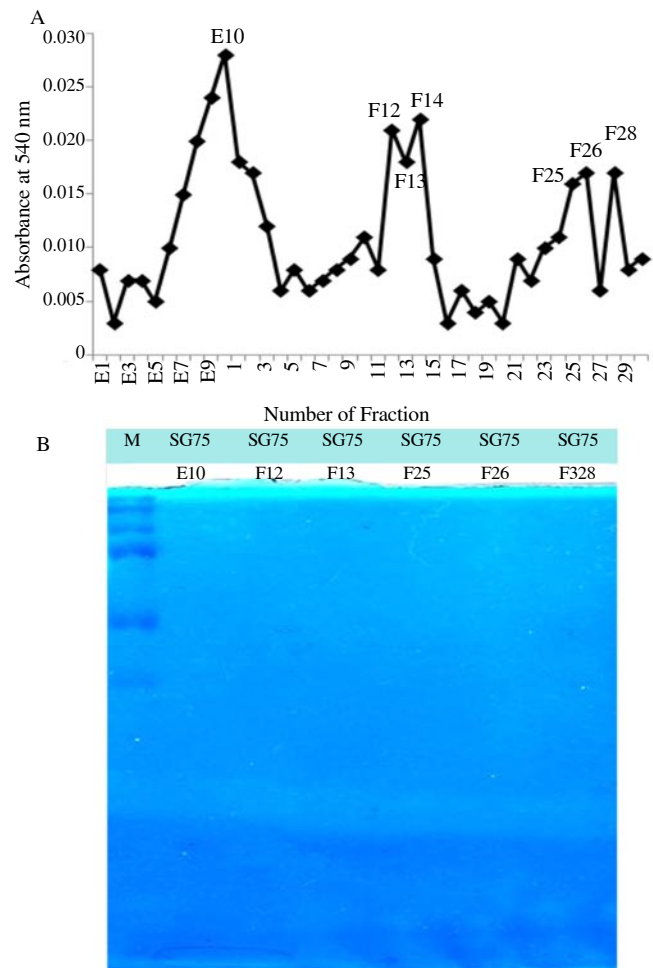


Figure 4. Sephadex-G75 chromatography profile and gelatin zymogram result.

A: Sephadex-G75 chromatography profile of Group A purified from DEAE-column chromatography; B: Gelatin zymogram result of protease activity in each fraction. M: Protein marker.

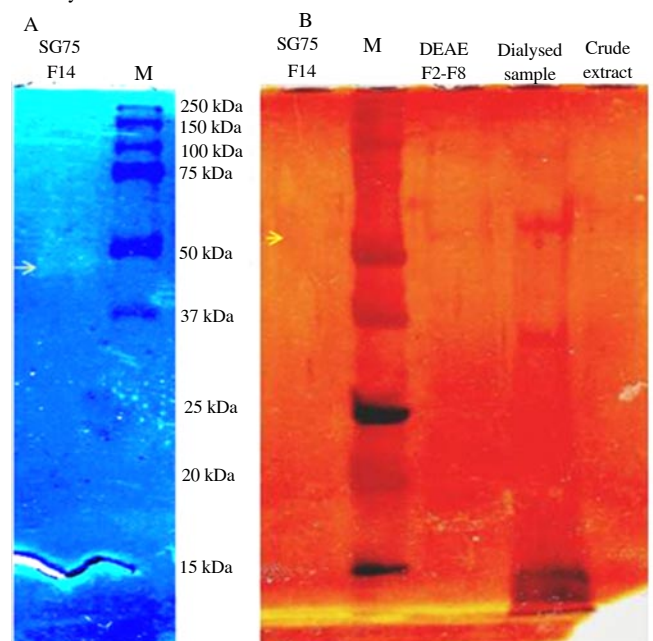


Figure 5. Gelatin zymogram and SDS-PAGE result. A: Gelatin zymogram of SG75-F14; B: SDS-PAGE result of SG75-F14, DEAE fractionation of Group A, dialyzed sample and unconcentrated crude extract. M: Protein marker.

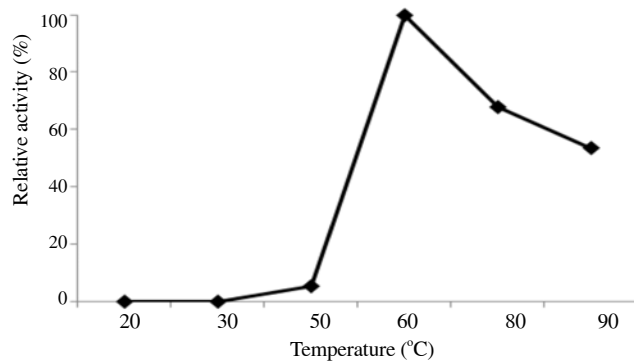
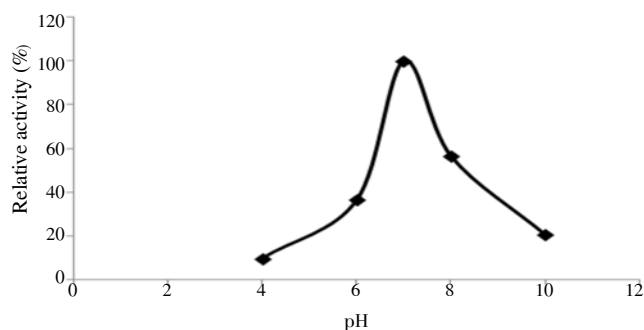
Table 1Summary of purification of extracellular protease *X. psidii* KT30.

Protease in different purification steps	Volume (mL)	Total activity (IU)	Protein (mg)	Specific activity (IU/mg)	Yield (%)	Purity (fold)
Crude extract	300.00	14.400	186.200	0.077	100.000	1.000
Ammonium sulfate	2.00	0.205	5.697	0.036	1.424	0.467
DEAE-Sepharose	1.00	0.013	0.245	0.054	0.090	0.702
Sephadex-SG75-F14	0.25	0.006	0.066	0.091	0.042	1.182

Protease activity was assayed at basal condition with temperature at 37 °C and pH of 7.4. The yield (%) of purified protein was calculated by dividing the total activity of protease in each fraction by total activity of protease in crude extract; meanwhile the purity was calculated by dividing specific protease activity in each fraction by specific protease activity in crude extract.

3.3. Characterization of extracellular protease of *X. psidii* KT30

Relationship between temperature and casein hydrolysis of protease is shown in Figure 6. The proteolytic activity was very low when enzyme was incubated at temperature ranging from 30 °C to 50 °C, but its activity rapidly increased and was maximum when exposed to 60 °C, but gradually decreased over the temperature range 80-90 °C. Meanwhile, dependency of protease activity on different pH was shown in Figure 7. Maximum value for protease activity was achieved at pH 7, low activity was recorded at either pH 4 or 10.

**Figure 6.** Optimum temperature of extracellular protease *X. psidii* KT30.**Figure 7.** Optimum pH of extracellular protease *X. psidii* KT30.

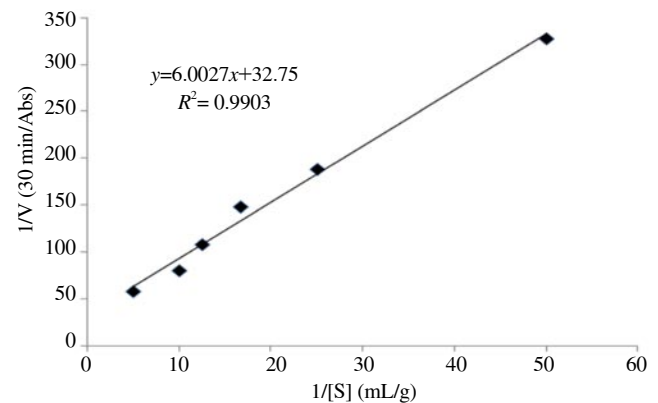
PMSF and EDTA were used to study the effect of protease inhibitors on protease activity. As pointed in Table 2, PMSF gave pronounced effect on enzyme activity compared to EDTA treatment. PMSF treatment brought about more than 70% on protease activity loss; meanwhile only 1 mmol/L EDTA treatment could bring more than

50% loss of protease activity. For kinetic parameters, as seen in Figure 8 the values of K_m and V_{max} for protease tested are 0.183 mg/mL and 7.01 µg/min, respectively.

Table 2Effect of protease inhibitors on activity of extracellular protease *X. psidii* KT30.

Inhibitors	Concentration (mmol/L)	Protease remaining activity (%)
PMSF	1.00	13.05±0.38
	0.04	22.54±0.29
EDTA	1.00	41.33±0.79
	0.04	61.99±4.90

Protease activity was assayed at maximum condition with temperature at 60 °C and pH of 7. Results are expressed as mean±SD ($n=3$).

**Figure 8.** Determination of kinetic parameters of extracellular protease *X. psidii* KT30 using Lineweaver-Burk plot.

Biochemical characteristics result as explained above showed that our protease has much similarity in several biochemical aspects with marine-fungal protease isolated from *Engyodontium album* (*E. album*) BTMFS10 as reported by Chellappan *et al.*, and the comparison was summarized in Table 3[20].

Table 3Comparison of marine-derived extracellular protease from *X. psidii* KT30 and *E. album* BTMFS10.

Biochemical aspects	<i>X. psidii</i> KT30	<i>E. album</i> BTMFS10
Sources	<i>Kappaphycus alvarezii</i> BRKA-1	Marine sediment
Molecular mass	71 kDa	28.1 kDa
Optimum pH	7	11
Optimum temperature	60 °C	60 °C
Type	Serine protease	Serine protease
Thermostability	Yes	Yes
Family of protease	Not determined	Serine protease (Family S8)
Inhibitor	PMSF	PMSF

4. Discussion

Extracellular protease has been successfully purified from supernatant of *X. psidii* KT30 at the 10th day. The 10-day culture was selected to isolate extracellular protease, in which the *X. psidii* KT30 was on early stationary phase. This phase was considered as the best stage to harvest the extracellular protease from some fungi with some degree of considerations, for instance, the influences of

external factors such as starvation condition, nutritional status and more importantly the availability of protein inducers could affect the production of extracellular proteases[21,22]. In our experiment, the protein inducers was absent in the medium for cultivating *X. psidii* KT30, but *X. psidii* KT30 could still produce protease comparable to other fungi that produce extracellular proteases; and interestingly, some particular fungal species producing extracellular proteases is specific protein inducers[11,23–26]. If the proteases production in *X. psidii* KT30 could also be modulated by protein inducers needs further clarification.

Extracellular protease of *X. psidii* KT30 had molecular mass of 43 kDa based on gelatin zymogram, but the molecular mass was twice bigger, 71 kDa, after separation on SDS-PAGE gel. This discrepancy is attributed to the difference of fundamental principle of protein separation between both methods. Reducing and boiling of protein sample in SDS-PAGE is critical to gain similar shape and ionic charge of proteins sample that lead to protein separation on the gel only based on their molecular mass. However, in zymogram, the protein sample was diluted in non-reducing buffer and boiling process was excluded prior to separation, so the proteins migration along the gel will depend not only on sizes but also on N-glycosylation and protein conformations which are almost affected by presence of disulfide bonding that reflects the active forms of protein. This phenomenon is also observed in other serine protease such as proteinase AtI from *Acremonium typhinum*[27], fungal Ser protease proteinase K[28], bacterial serine protease subtilisin and engineered subtilisin E from *Bacillus subtilis*[29,30]. Furthermore, oligomeric status of protein was proven by single clear zone formation after renaturation in gelatin zymography, indicating that extracellular protease of *X. psidii* KT30 has monomeric structure. Some other fungal serine protease such as extracellular protease PoSI and subtilisin like serine protease Eap also showed similarity on structure, showing mature peptide at their monomeric enzyme state[31,32].

Extracellular protease of *X. psidii* KT30 in its native state has molecular mass smaller than 205 kDa of serine protease isolated from *Acremonium typhinum*[27]. However, molecular mass in its denatured state was in range of molecular mass of extracellular serine protease purified from *E. album* (28.1 kDa)[20], *Lecanicillium psalliotae* (32 kDa)[26], *Hirsutella rhossiliensis* OWVT-1 (33 kDa)[11], *Dactylella shizishanna* (35 kDa)[20], *Purpureocillium lilacinum* (37 kDa)[33], *Aspergillus fumigatus* (88 kDa)[18], and *Paracoccidioides brasiliensis* (66 kDa)[34]; all these exhibit numerous biological activities, or some are important in field of biotechnological applications.

The factors such as temperature, pH and protease inhibitor influence the activity of enzymes. The optimum temperature for activity of extracellular protease of *X. psidii* KT30 is 60 °C. The protease activity still remained above 50% when temperature raised

to 90 °C, indicating that it was moderately thermostabile protease. Proteolytic activity was highest at pH 7 but low either at pH of 4 or 10. Surprisingly, the activity of enzyme remained above 50% when pH was increased from 7 to 8. Furthermore, PMSF has tremendous effect on protease activity; meanwhile EDTA gave only mild effect. The above mentioned indicated that this enzyme was thermostabile neutral serine protease type. The biochemical properties of protease produced by *X. psidii* KT30 are nearly the same as what found in 88 kDa serine protease extracted from *Aspergillus fumigatus* in terms of temperature 60 °C, pH 7 and kinds of protease inhibitors (mostly inhibited by PMSF but not by EDTA) for optimum enzymatic activity[19].

The kinetic parameters such as K_m and V_{max} are the essential factors to predict behavior of enzymes in catalytic process of their substrates. Using Lineweaver-Burk plot, we had predicted that the value of K_m and V_{max} of extracellular protease *X. psidii* KT30 are 0.183 mg/mL and 7.01 $\mu\text{g}/\text{min}$ ($\sim 233.67 \mu\text{g}/\text{min}/\text{mL}$) and those values are lower than those K_m (8.26 mg/mL) and V_{max} (668 $\mu\text{g}/\text{min}/\text{mL}$) of serine protease from *Termitomyces albuminosus*[35].

Protease enzymes particularly with thermostabile property have been used widely for their usefulness in biotechnological and biomedical applications. This present research is the first study reporting about proteases isolated from marine-derived endophytic fungi and its characterization. Our further study will also focus on the characterization of protease gene to gain more information about the function of this fungal protease in *X. psidii* KT30 growth and development.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Isolation and characterization of bioactive compounds with potential applications in preventing human diseases is one of the most attracting topics. Endophytic fungi are known to produce several compounds with antibacterial, antitumoral and antiviral properties. Therefore there is a growing interest in exploring new sources of bioactive molecules.

Research frontiers

The extracellular protease secreted by *X. psidii* KT30 was isolated and purified using three steps of protein purification (ammonium sulfate precipitation, DEAE-sepharose column chromatography, and sephadex SG75 column chromatography). Qualitative and quantitative enzymatic assays (*i.e.* direct protease assay on solid

culture medium, screening on gelatin-agarose plates, protease activity measurements at 540 nm) were used to characterize the purified protease. Its molecular mass was also determined and the optimum temperature, pH and the effects of protease inhibitors (PMSF and EDTA) were assayed, showing that the molecule has maximum activity at 60 °C and pH 7 and belongs to serine-proteases group.

Related reports

Several examples of extracellular proteases produced from other fungal species are reported by other researchers. A comparison between the biochemical properties of marine-derived extracellular protease from *X. psidii* KT30 and *E. album* BTMFS10 reported in other study is also performed in this present study.

Innovations and breakthroughs

The aim of this study is to isolate the main active molecule responsible of biological activity in *X. psidii*, since the ethyl acetate extract of *X. psidii* KT30 broth culture was known to have antiviral, antibacterial, and anticancer activity. Although current literature refers on protease isolation from marine-derived endophytic fungi, this is the first study reporting about its isolation and characterization from *X. psidii* KT30.

Applications

The subject of the present study is attractive because most of current researches focus on secondary metabolites, while exploring endophytic fungi as a source of active compounds could be of greater interest since protein biomolecules are more save compared to secondary metabolites when applied as drugs or food preservatives in term of their less toxicity. Particularly the thermostable properties of the isolated molecule are interesting for its biomedical applications.

Peer review

This paper is an interesting study focusing on the extraction and biochemical characterization of an extracellular protease produced by the marine fungus *X. psidii* KT30. The biochemical properties displayed by this molecule suggest its promising application in the field of biotechnology or medicine, obviously following further experimentation.

References

- [1] Strobel GA. Endophytes as sources of bioactive products. *Microbes Infect* 2003; **5**: 535-544.
- [2] Kusari S, Hertweck C, Spiteller M. Chemical ecology of endophytic fungi: origin of secondary metabolites. *Chem Biol* 2012; **19**: 792-798.
- [3] Kim SK, Dewapriya P. Anticancer potentials of marine-derived fungal metabolites. In: Kim SW, editor. *Marine microbiology: bioactive compounds and biotechnological applications*. 1st ed. Weinheim: Wiley-VCH; 2013, p. 237-243.
- [4] Tarman K, Lindequist U, Wende K, Porzel A, Arnold N, Wessjohann LA. Isolation of a new natural product and cytotoxic and antimicrobial activities of extracts from fungi of Indonesian marine habitats. *Mar Drugs* 2011; **9**: 249-306.
- [5] Hu QX, Zhang GQ, Zhang RY, Hu DD, Wang HX, Ng TB. A novel aspartic protease with HIV-1 reverse transcriptase inhibitory activity from fresh fruiting bodies of the wild mushroom *Xylaria hypoxylon*. *J Biomed Biotechnol* 2012; doi: 10.1155/2012/728975.
- [6] Park BT, Na KH, Jung EC, Park JW, Kim HH. Antifungal and anticancer activities of a protein from mushroom *Cordyceps militaris*. *Korean J Physiol Pharmacol* 2009; **13**: 49-54.
- [7] Sun J, Zhao Y, Chai H, Wang H, Ng TB. A novel alkaline protease with antiproliferative activity from fresh fruiting bodies of the toxic wild mushroom *Amanita farinosa*. *Acta Biochim Pol* 2011; **58**: 567-572.
- [8] Schulz B, Boyle C, Draeger S, Rommert AK, Krohn K. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycol Res* 2002; **106**: 996-1004.
- [9] Schumacher M, Kelkel M, Dicato M, Diederich M. Gold from the sea: marine compounds as inhibitors of the hallmarks of cancer. *Biotechnol Adv* 2011; **29**: 531-547.
- [10] Ng TB. Peptides and proteins from fungi. *Peptides* 2004; **25**: 1055-1073.
- [11] Wang B, Liu X, Wu W, Liu X, Li S. Purification, characterization, and gene cloning of an alkaline serine protease from a highly virulent strain of the nematode-endoparasitic fungus *Hirsutella rhossiliensis*. *Microbiol Res* 2009; **164**: 665-673.
- [12] Hassan MA, Haroun BM, Amara AA, Serour EA. Production and characterization of keratinolytic protease from new wool-degrading *Bacillus* species isolated from Egyptian ecosystem. *Biomed Res Int* 2013; doi: 10.1155/2013/175012.
- [13] More SS, Renuka PS, Pruthvi K, Swetha M, Malini S, Veena SM. Isolation, purification and characterization of fungal laccase from *Pleurotus* sp. *Enzyme Res* 2011; doi:10.4061/2011/248735.

- [14] Cupp-Enyard C. Sigma's non-specific protease activity assay casein as a substrate. *J Vis Exp* 2008; doi: 10.3791/899.
- [15] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685.
- [16] Raser KJ, Posner A, Wang KK. Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch Biochem Biophys* 1995; **319**(1): 211-216.
- [17] Kleiner DE, Stetler-Stevenson WG. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal Biochem* 1994; **218**: 325-329.
- [18] Hernández-Martínez R, Gutiérrez-Sánchez G, Bergmann CW, Loera-Corral O, Rojo-Dominguez A, Huerta-Ochoa S, et al. Purification and characterization of a thermodynamic stable serine protease from *Aspergillus fumigatus*. *Process Biochem* 2011; **46**: 2001-2006.
- [19] Zhang XQ, Liu QH, Zhang GQ, Wang HX, Ng TB. Purification and molecular cloning of a serine protease from mushroom *Hypsizygus marmoreus*. *Process Biochem* 2010; **45**: 724-730.
- [20] Chellappan S, Jasmin C, Basheer SM, Kishore A, Elyas KK, Bhat SG, et al. Characterization of an extracellular alkaline serine protease from marine *Engyodontium album* BTMFS10. *J Ind Microbiol Biotechnol* 2011; **38**: 743-752.
- [21] Valueva TA, Kudryavtseva NN, Gvozdeva EL, Sof'in AV, Il'ina NY, Pobedinskaya MA, et al. Serine proteinases secreted by two isolates of the fungus *Alternaria solani*. *J Basic Appl Sci* 2013; **9**: 105-115.
- [22] Tunlid A, Rosen S, Ek B, Rask L. Purification and characterization of an extracellular serine protease from the nematode-trapping fungus *Arthrobotrys oligospora*. *Microbiology* 1994; **140**: 1687-1695.
- [23] Bonants PJ, Fitters PF, Thijs H, den Belder E, Waalwijk C, Henfling JW. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. *Microbiology* 1995; **141**: 775-784.
- [24] Segers R, Butt TM, Kerry BR, Peberdy JF. The nematophagous fungus *Verticillium chlamydosporium* produces a chymoelastase-like protease which hydrolyses host nematode proteins *in situ*. *Microbiology* 1994; **140**: 2715-2723.
- [25] Yang J, Huang X, Tian B, Wang M, Niu Q, Zhang K. Isolation and characterization of a serine protease from the nematophagous fungus, *Lecanicillium psalliotae*, displaying nematocidal activity. *Biotechnol Lett* 2005; **27**: 1123-1128.
- [26] Lindstrom JT, Belanger FC. Purification and characterization of an endophytic fungal proteinase that is abundantly expressed in the infected host grass. *Plant Physiol* 1994; **106**: 7-16.
- [27] Kolvenbach CG, Narhi LO, Lazenby K, Samal B, Arakawa T. Comparative study on proteinase R, T, and K from *Tritirachium album* limber. *Int J Pept Protein Res* 1990; **36**: 387-391.
- [28] Narhi LO, Arakawa T. Sodium dodecyl sulfate polyacrylamide gel electrophoresis as a method for studying the stability of subtilisin. *Biochim Biophys Acta* 1989; **990**: 144-149.
- [29] Takagi H, Takahashi T, Momose H, Inouye M, Maeda Y, Matsuzawa H, et al. Enhancement of the thermostability of subtilisin E by introduction of a disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. *J Biol Chem* 1990; **265**: 6874-6878.
- [30] Faraco V, Palmieri G, Festa G, Monti M, Sannia G, Giardina P. A new subfamily of fungal subtilases: structural and functional analysis of a *Pleurotus ostreatus* member. *Microbiology* 2005; **151**: 457-466.
- [31] Jasmin C, Chellappan S, Sukumaran RK, Elyas KK, Bhat SG, Chandrasekaran M. Molecular cloning and homology modelling of a subtilisin-like serine protease from the marine fungus, *Engyodontium album* BTMFS10. *World J Microbiol Biotechnol* 2010; **26**: 1269-1279.
- [32] Simkovic M, Kurucova A, Hunova M, Varecka L. Induction of secretion of extracellular proteases from *Trichoderma viride*. *Acta Chim Slovaca* 2008; **1**(1): 250-264.
- [33] Cavello IA, Hours RA, Rojas NL, Cavalitto SF. Purification and characterization of a keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876. *Process Biochem* 2013; **48**: 972-978.
- [34] Parente JA, Salem-Izacc SM, Santana JM, Pereira M, Borges CL, Bailao AM, et al. A secreted serine protease of *Paracoccidioides brasiliensis* and its interactions with fungal proteins. *BMC Microbiol* 2010; doi: 10.1186/1471-2180-10-292.
- [35] Zheng S, Wang H, Zhang G. A novel alkaline protease from wild edible mushroom *Termitomyces albuminosus*. *Acta Biochim Pol* 2011; **58**(2): 269-273.