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PURIFICATION AND PRELIMINARY CHARACTERIZATION OF A COLD-ADAPTED EXTRACELLULAR PROTEINASE FROM *TRICHODERMA ATROVIRIDE*

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Running title: Cold-adapted proteinase from *Trichoderma atroviride*

ABSTRACT

Eleven cold-tolerant *Trichoderma* isolates were screened for the production of proteolytic activities at 10 °C. Based on the activity profiles determined with paranitroanilide substrates at 5 °C, strain T221 identified as *Trichoderma atroviride* was selected for further investigations. The culture broth of the strain grown at 10 °C in casein-containing culture medium was concentrated by lyophilization and subjected to gel filtration, which was followed by chromatofocusing of the fraction showing the highest activity on *N*-benzoyl-Phe-Val-Arg-paranitroanilide. The purified enzyme had a molecular weight of 24 kDa, an isoelectric point of 7.3 and a pH optimum of 6.2. The temperature optimum of 25 °C and the low thermal stability suggested that it is a true cold-adapted enzyme. Substrate specificity data indicate that the enzyme is a proteinase with a preference for Arg or Lys at the P1 position. The effect of proteinase inhibitors suggests that the enzyme has a binding pocket similar to the one present in trypsin.

Keywords: cold tolerance – psychrophylic enzymes – *Trichoderma* – trypsin-like proteinase

INTRODUCTION

Members of the genus *Trichoderma* are asexual, soil-inhabiting filamentous fungi with teleomorphs belonging to the genus *Hypocrea* (Ascomycota, Pyrenomycetes, Hypocreales, Hypocreaceae). Certain *Trichoderma* species are well-known as cellulase producers of biotechnological importance [17], while other representatives of the genus are effective antagonists of plant pathogenic fungi, thus being potential candidates for biological control. Proposed mechanisms of antagonism include competition for space and nutrients, antibiosis, facilitation of seed germination and growth of the plants, induction of plant defense responses and mycoparasitism by the action of cell-wall degrading enzymes [3, 8, 13]. Although the involvement of extracellular chitinolytic and β-1,3-glucanolytic enzyme systems in the mycoparasitism of *Trichoderma* has been studied in details [10, 22], the extracellular proteolytic enzyme system remained relatively unknown in the case of this genus. Fortunately, in the recent years more and more attention is focused on the investigation of *Trichoderma* proteinases and their potential role in biological control [9, 19].

Thermal environments in temperate soils may be around 10 °C at 5–10 cm in September and can rapidly cool to just above freezing by mid-October [14]. As most of the *Trichoderma* strains are mesophilic, they can not protect germinating seeds from soilborne diseases caused by cold-tolerant strains of plant pathogenic fungi at low temperatures. Only a few studies have addressed the effect of temperature on the interaction between *Trichoderma* as a biocontrol agent and the targeted plant pathogens. Antal et al. [2] selected cold-tolerant *Trichoderma* strains capable of considerable growth on artificial media even at 5 °C. These cold-tolerant isolates inhibited the

mycelial growth of plant pathogenic fungi and overgrew them in confrontation assays performed at 10 °C. Clarkson et al. [4] have found that *T. viride* destroyed about 40% of *Sclerotium cepivorum* sclerotia at 10 °C after 8 weeks and suggested that further degradation may occur at both 10 °C and 5 °C with longer incubation. *Trichoderma* strains with such capabilities are promising candidates for biological control during cold seasons therefore the investigation of their extracellular enzymes supposed to be involved in mycoparasitism is of great importance. In the present study we report the purification and characterization of a psychrophilic (cold-adapted) proteinase from a cold-tolerant *Trichoderma* strain.

MATERIALS AND METHODS

Strains and culture conditions

Cold-tolerant *Trichoderma* strains involved in the study were T66, T114, T221, T228 and T334 isolated from forest soil [2] as well as TB17, TB37, TB69, TB75, TB94 and TB103 isolated from the roots of winter wheat [20]. All isolates derived from South East Hungary were maintained on minimal medium (5 g l^{-1} glucose, 5 g l^{-1} $(NH_4)_2SO_4$, 1 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSO_4$ and 15 g l^{-1} agar]. The strains were grown at 10 °C on a shaker at 200 rpm in flasks of 700 ml volume containing 200 ml of a casein-containing medium inductive for proteinases (1 g l^{-1} $MgSO_4$, 1 g l^{-1} $NaNO_3$, 1 g l^{-1} KH_2PO_4, 1 g l^{-1} Na_2SO_4, 1 g l^{-1} mannitol and 1 g l^{-1} skim milk powder). After seven days of culturing, the culture broths were collected by filtration and kept frozen at −20 °C until screening for proteolytic activities.

Screening for proteolytic activities

Proteolytic activities were assayed with the paranitroanilide (pNA) substrates *N*-benzoyl-Phe-Val-Arg-pNA and *N*-succinyl-Ala-Ala-Pro-Phe-pNA (Sigma-Aldrich). In the wells of a microtiter plate, 50 µl of each culture broth was incubated for 30 minutes at 5°C with 50 µl substrate (1 mg ml^{-1} in dimethyl-sulfoxide) in a final volume of 150 µl buffered to pH 6.5 with Sørensen phosphate buffer. The optical density of the samples was determined with a Uniskan II microtiter plate spectrophotometer (Labsystems, Helsinki, Finland) at a wavelength of 405 nm. Enzyme activities were expressed in unit (u), 1 u is defined as the activity that releases 1 nmol paranitroaniline in 1 min under the assay conditions.

Molecular identification of strain T221

DNA-isolation, PCR-amplification of the internal transcribed spacer (ITS) region and automatic DNA sequencing of the fragments were carried out as described previously [16]. ITS sequence analysis was performed with *TrichO*key 1.0 (http://www.isth.info/) based on an oligonucleotide barcode: a diagnostic combination of several oligonucleotides (hallmarks) specifically allocated within the ITS 1 and 2 sequences of the ribosomal DNA repeat [5].

Purification of a proteinase from strain T221

The culture broth was concentrated 40-fold by lyophilization and 10 ml was applied to a Sephadex G-100 chromatography column (Pharmacia) of 3.34 X 36 cm dimensions. A total number of 55 fractions (4.5 ml each) were collected at a flow rate of 1.5 ml min^{-1}, the eluent contained 1 g l^{-1} NaCl. Trypsin-like activities were measured at 5 °C in fractions 1–30 with *N*-benzoyl-Phe-Val-Arg-pNA as described above. The fraction showing the highest activity was concentrated on a membrane-filter and subjected to chromatofocusing on a 12 cm Polybuffer exchanger 94 (Sigma) gel bed prepared in 3 μg ml^{-1} histidin (pH 7.0) and eluted with Polybuffer 74. A total number of 60 fractions (1.5 ml each) were collected and their proteolytic activities were measured with both *N*-benzoyl-Phe-Val-Arg-pNA and *N*-succinyl-Ala-Ala-Pro-Phe-pNA. Fractions 39-46 with the highest activities on *N*-benzoyl-Phe-Val-Arg-pNA were pooled and concentrated 3-fold by lyophilization for the further characterization of the enzyme.

Characterization of the purified enzyme

The molecular weight of the enzyme was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) carried out for 50 minutes at 200 V in NuPAGE MOPS running buffer on a 4-12% NuPAGE Bis-Tris precast gel (Invitrogen) run in an XCELL vertical gel electrophoresis system (Novex, San Diego, CA, USA). The marker SeeBlue Plus2 (Invitrogen) was used for the determination of the molecular weight of the enzyme. The isoelectric point was determined by isoelectric focusing on a pH 3–10 vertical precast IEF gel (Invitrogen) run at 5 °C for 1 hour at 100 V followed by 1 hour at 200 V and finally 1 hour at 500 V in an XCELL vertical gel electrophoresis system. Novex IEF cathode, anode and sample buffers were applied for isoelectric focusing

according to the manufacturer's instructions. IEF-Marker pI 3.6–9.3 (Fluka) was used as the pI standard. The gels were stained with the EZBlue gel staining reagent (Sigma-Aldrich).

Temperature dependence was examined by activity measurements after 1 h of incubation with *N*-benzoyl-Phe-Val-Arg-pNA at 9 temperature values in the range of 5–45 °C on a chilling/heating block for microtiter plates (Cole-Parmer Instruments Company, Vernon Hills, IL, USA). The thermal stability of the enzyme was examined after preincubation at 40, 50, 60 and 70 °C for 60 min by the measurement of the residual activity at 25 °C as described above. pH-dependence was measured in the pH range between 5.0 and 8.0 adjusted with Sørensen phosphate buffer and the enzyme activities were measured at 25 °C. The substrate specificity of the purified proteinase was examined with the chromogenic substrates listed in Table 1 (all from Sigma-Aldrich). Each examined substrate was tested at an end concentration of 1 mM, measurements were performed after 30 min of incubation at 25 °C. The inhibition effects of the inhibitors listed in Table 2 on the proteinase activity were determined by the measurement of the residual activity after preincubation with the inhibitors (end concentrations given in Table 2) at 25 °C for 1 h. All measurements were carried out in three replicates, the standard deviation values were determined.

RESULTS

Screening of Trichoderma *strains for cold adapted proteolytic activities*

Proteolytic activities were measured at 5 °C from the culture broth of cold-tolerant *Trichoderma* strains grown in casein-containing medium at 10 °C. Strain T221 exhibited the highest trypsin-like (5.76 u/ml) and chymotrypsin-like activities (6.29 u/ml) cleaving the substrates *N*-benzoyl-Phe-Val-Arg-pNA and *N*-succinyl-Ala-Ala-Pro-Phe-pNA, respectively. Based on our previously recorded data, the radial extension rates of the examined cold tolerant isolates ranged from 1.72±0.11 to 2.04±0.04 mm/day on yeast extract agar medium at 5°C. Although strain T221 could be characterized with the best growth capabilities (2.04±0.04 mm/day), the differences between the radial extension rates of the isolates were modest when compared with the differences between their proteolytic activities measured. Trypsin-like and chymotrypsin-like activities of T221 were 1.9-5.5 and 1.7-9.0 times higher, respectively, than those of the other cold tolerant strains, suggesting that T221 has the highest specific proteolytic activity, therefore this strain was selected for further investigations.

Identification of strain T221

Strain T221 was originally identified as *T. aureoviride* using the key of Rifai [15]. However, since this morphology-based identification, *T. aureoviride* and its teleomorph *Hypocrea aureoviridis* have been redefined as rare species restricted to the UK and the Netherlands, as the molecular characters of most strains originally identified as *T. aureoviride* proved to be different from those of the ex-type [11]. Based on the analysis of ITS 1 and 2 sequences (GenBank accession no. **AY585878**) with *TrichO*key

1.0, the strain proved to belong to *T. atroviride*, a species of section *Trichoderma*, clade *Rufa* according to the currently accepted species concept of the genus [6].

Purification of a proteinase from T. atroviride *T221*

The purification process is presented in Fig. 1. A single, sharp peak of proteolytic activities cleaving *N*-benzoyl-Phe-Val-Arg-pNA at 5 °C could be observed after gel filtration chromatography, with maximal activity in fraction 20 (Fig. 1A). As a previous study reported that *Trichoderma* strains produce chymotrypsin-like activities in the same molecular weight range as that of trypsin-like activities [1], fraction 20 from gel filtration was further purified by chromatofocusing. Activity in the chromatofocused fractions was assayed for substrates *N*-benzoyl-Phe-Val-Arg-pNA and *N*-succinyl-Ala-Ala-Pro-Phe-pNA. Maximal trypsin-like and chymotrypsin-like activities were detected in fractions 42 (3.1 u/ml) and 11 (1.4 u/ml), respectively, indicating that chromatofocusing clearly separates these two types of activities (Fig. 1B).

Characterization of the purified enzyme

The molecular weight of the enzyme proved to be about 24 kDa by SDS PAGE, the isoelectric point was estimated at 7.3. The temperature optimum was detected at 25 °C (Fig. 2), which supports the idea that this cold-tolerant *Trichoderma* strain is able to secrete a true psychrophilic proteinase. The enzyme was completely inactivated after 1 h of preincubation at all tested temperatures except for 40 °C (49% residual activity), indicating high thermal instability. The pH optimum of the proteinase proved to be 6.2.

Substrate specificity data indicate the preference of the purified enzyme for a positively charged polar residue (Arg or Lys) at the carboxyl side of the cleaved bond, as expected for a trypsin-like proteinase (Table 1). Substrates for elastases and chymotrypsin were not hydrolyzed. The enzyme was inhibited by leupeptin and TLCK, as well as by benzamidine indicating the presence of a binding pocket for Arg or Lys, similar to the one present in trypsin (Table 2). The examined inhibitors of chymotrypsin-like serine proteinases (TPCK), metalloproteinases (EDTA) and cystein proteinases (*N*-ethyl-maleimide) did not influence the enzymatic activity. Mercury strongly inhibited the enzyme, while copper caused significant enhancement of the activity.

DISCUSSION

Data available about the purification and characterization of proteolytic enzymes including acidic aspartyl proteinases, subtilisin-like serine proteinases as well as trypsin-like serine proteinases produced by *Trichoderma* species have been reviewed by Kredics et al. [9]. In the present study, the combination of gel filtration on a Sephadex G-100 column and chromatofocusing on a Polybuffer exchanger 94 gel bed has been applied for the purification of a proteinase to electrophoretic homogeneity from the culture broth of the cold-tolerant strain *T. atroviride* T221.

The molecular weight of the isolated enzyme proved to be about 24 kDa, similar to those of the trypsin-like serine proteinases purified from *T. harzianum* by Suarez et al. [18] (28 kDa) and from *T. viride* by Uchikoba et al. [21] (25 kDa). These two previously purified enzymes were characterized with pI values of 4.8 and 7.3,

respectively, the latter is in congruence with the isoelectric point of the proteinase described here.

The thermodependence and stability of the purified proteinase exhibited the characteristics described for cold-adapted enzymes, i.e. high activity in the low temperature range, a maximal activity shifted towards lower temperatures and weak thermostability resulting from improved flexibility [7]. Where reported, the temperature values optimal for other *Trichoderma* proteinases were found to be higher, between 35 and 50 °C [9, 18].

To our knowledge, the present study is the first report about the purification of a cold-adapted proteinase from the genus *Trichoderma*. Previous studies provided data about the extracellular enzyme activities of cold tolerant *Trichoderma* strains [2, 9]. Besides proteinases, they have been shown to secrete also chitinolytic and cellulolytic activities that are functional at 5 °C [2]. Psychrotrophic soil microorganisms are supposed to employ enzyme systems with a range of temperature optimized isoenzymes [12]. Psychrophilic enzymes may therefore play an important role in the adaptation of cold-tolerant strains belonging to the otherwise mesophilic genus *Trichoderma*. Furthermore, the ability to produce cold-adapted extracellular hydrolases makes cold-tolerant *Trichoderma* strains promising candidates for the biological control of plant pathogenic fungi at lower temperatures.

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REFERENCES

1. Antal, Z., Kredics, L., Manczinger, L., Ferenczy, L. (2001) Extracellular enzyme profiles of mycoparasitic *Trichoderma* strains. *IOBC/WPRS Bull. 24(3),* 337–340.

2. Antal, Z., Manczinger, L., Szakács, G., Tengerdy, R. P., Ferenczy, L. (2000) Colony growth, *in vitro* antagonism and secretion of extracellular enzymes in cold-tolerant strains of *Trichoderma* species. *Mycol. Res. 104,* 545–549.

3. Benítez, T., Rincón, A. M., Limón, M. C., Codón A. C. (2004) Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol. 7,* 249−260.

4. Clarkson, J. P., Mead, A., Payne, T., Whipps, J. M. (2004) Effect of environmental factors and *Sclerotium cepivorum* isolate on sclerotial degradation and biological control of white rot by *Trichoderma. Plant Pathol. 53,* 353–362.

5. Druzhinina, I., Kopchinskiy, A. G., Komon, M., Bissett, J., Szakacs, G., Kubicek, C. P. (2005) An oligonucleotide barcode for species identification in *Trichoderma* and *Hypocrea*. *Fungal Genet. Biol. 42,* 813−828.

6. Druzhinina, I., Kubicek, C. P. (2005) Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? *J. Zhejiang Univ. Sci. 6B,* 100–112.

7. Feller, G. (2003) Molecular adaptations to cold in psychrophilic enzymes. *Cell. Mol. Life Sci. 60,* 648–662.

8. Howell, C. R. (2003) Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Dis. 87,* 4–10.

9. Kredics, L., Antal, Z., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyi, C., Nagy, E. (2005) Extracellular proteases of *Trichoderma* species – a review. *Acta Microbiol. Immunol. Hung. 52,* 169–184.

10. Kubicek, C. P., Mach, R. L., Peterbauer, C. K., Lorito, M. (2001) *Trichoderma*: from genes to biocontrol. *J. Plant Pathol. 83(S2),* 11–23.

11. Lieckfeldt, E., Kullnig, C. M., Kubicek, C. P., Samuels, G. J., Börner, T. (2001) *Trichoderma aureoviride*: phylogenetic position and characterization. *Mycol. Res. 105,* 313–322.

12. Loveland, J., Gutshall, K., Kasmir, J., Prema, P., Brenchley, J. E. (1994) Characterisation of psychrotropic microorganisms producing β-galactosidase activities. *Appl. Environ. Microbiol. 60,* 12–28.

13. Naár, Z., Kecskés, M. (1998) Factors influencing the competitive saprophytic ability of *Trichoderma* species. *Microbiol. Res. 153,* 119–129.

14. Payne, D., Gregory, P. J. (1988) The temperature of the soil. In: Wild, E. (ed.) Russel's Soil Conditions and Plant Growth, 11th edn. Longman Scientific and Technical, Harlow, U.K., pp. 282–297.

15. Rifai, M. A. (1969) A revision of the genus *Trichoderma*. *Mycol. Pap. 116,* 1–56.

16. Rigó, K., Varga, J., Tóth, B., Mesterházy, Á., Kozakiewicz, Z. (2002) Phylogenetic analysis of *Aspergillus* section Flavi based on sequences of the internal transcribed spacer regions and the 5.8 S rRNA gene. *J. Gen. Appl. Microbiol.* 48, 9–16.

17. Schmoll, M., Kubicek, C. P. (2003) Regulation of *Trichoderma* cellulase formation: lessons in molecular biology from an industrial fungus. *Acta Microbiol. Immunol. Hung. 50,* 125–140.

18. Suarez, B., Rey, M., Castillo, P., Monte, E., Llobell, A. (2004) Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. *Appl. Microbiol. Biotechnol. 65,* 46–55.

19. Szekeres, A., Kredics, L., Antal, Z., Manczinger, L., Kevei, F. (2004) Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum. FEMS Microbiol. Lett. 233,* 215–222.

20. Szekeres, A., Láday, M., Kredics, L., Antal, Z., Hatvani, L., Manczinger, L. (2004) Investigation of *Trichoderma* strains isolated from winter wheat rhizosphere. *IOBC/WPRS Bull. 27(8),* 155–158.

21. Uchikoba, T., Mase, T., Arima, K., Yonezawa, H., Kaneda, M. (2001) Isolation and characterization of a trypsin-like protease from *Trichoderma viride. Biol. Chem. 382,* 1509–1513.

22. Vazquez-Garciduenas, S., Leal-Morales, C. A., Herrera-Estrella, A. (1998) Analysis of the β-1,3-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol. 64,* 1442–1446.

Table 1

Substrate specificity of the purified proteinase

Substrate	**Relative activity (%)**[a]
N-benzoyl-Phe-Val-Arg-pNA	100
N-benzoyl-L-Arg-pNA	65
N-benzoyl-Pro-Phe-Arg-pNA	30
N-benzoyl-Val-Gly-Arg-pNA	24
Np-tosyl-Gly-Pro-Arg-pNA	180
Np-tosyl-Gly-Pro-Lys-pNA	63
N-succinyl-Ala-Ala-Pro-Phe-pNA	0
N-succinyl-Ala-Ala-Ala-pNA	3
N-succinyl-Ala-Ala-Pro-Leu-pNA	2
N-CBZ-Ala-Ala-Leu-pNA	0

[a]relative activities are presented in the percentage of the activity on *N*-benzoyl-Phe-Val-Arg-pNA

Table 2

Effects of various compounds on the proteinase purified from *T. atroviride*

Inhibitor	**End concentration (mM)**	**Relative activity (%)**
None	-	100.0
TLCK	10.0	5.6
TPCK	10.0	100.3
Leupeptin	1.0	3.2
Benzamidine	2.0	20.0
PMSF	10.0	43.9
EDTA	1.0	100.4
N-ethyl-maleimide	1.0	93.0
2-mercaptoethanol	1.0	95.4
$HgCl_2$	1.0	10.1
$CuSO_4$	1.0	124.0

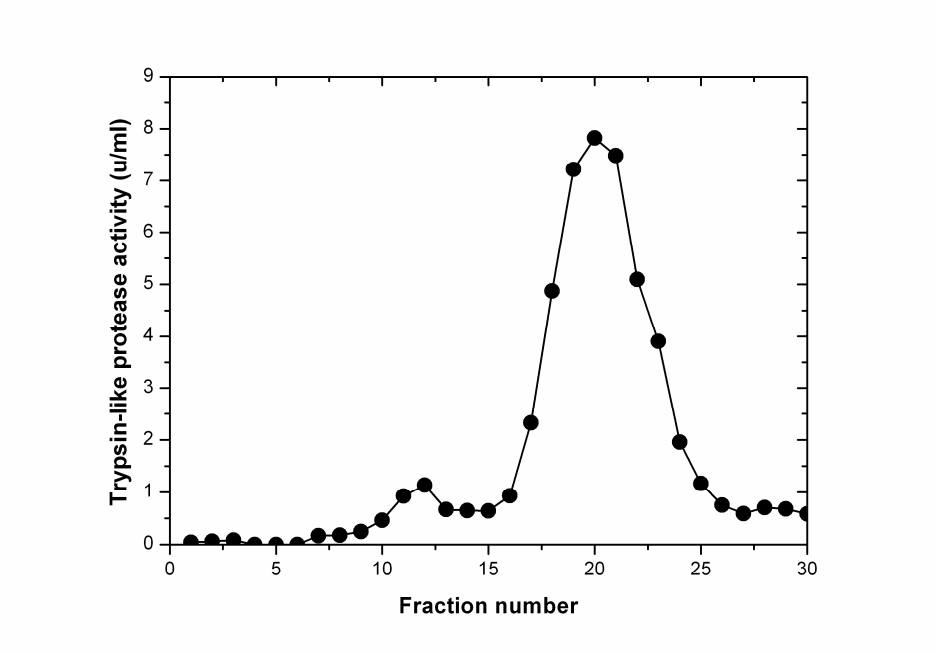
TLCK: tosyl-L-lysine chloromethyl ketone, TPCK: tosyl-L-phenylalanine chloromethyl ketone, PMSF: phenylmethyl-sulphonylfluoride

Figure legends:

Fig. 1. Purification of a proteinase from *T. atroviride* T221 by gel filtration (A) and chromatofocusing (B).

Fig. 2. Temperature dependence of the purified proteinase. Error bars indicate standard deviation.

A.


Trypsin-like protease activity (u/ml)
Fraction number
0
1
2
3
4
5
6
7
8
9
0
5
10
15
20
25
30

B.

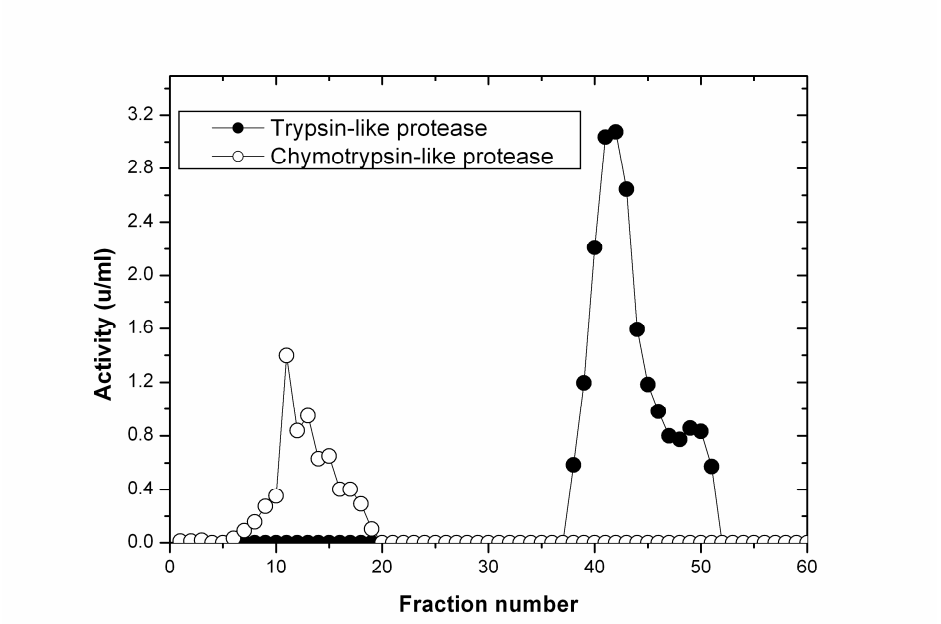

Trypsin-like protease
Chymotrypsin-like protease
Activity (u/ml)
Fraction number
0.0
0.4
0.8
1.2
1.6
2.0
2.4
2.8
3.2
0
10
20
30
40
50
60

Fig. 1.

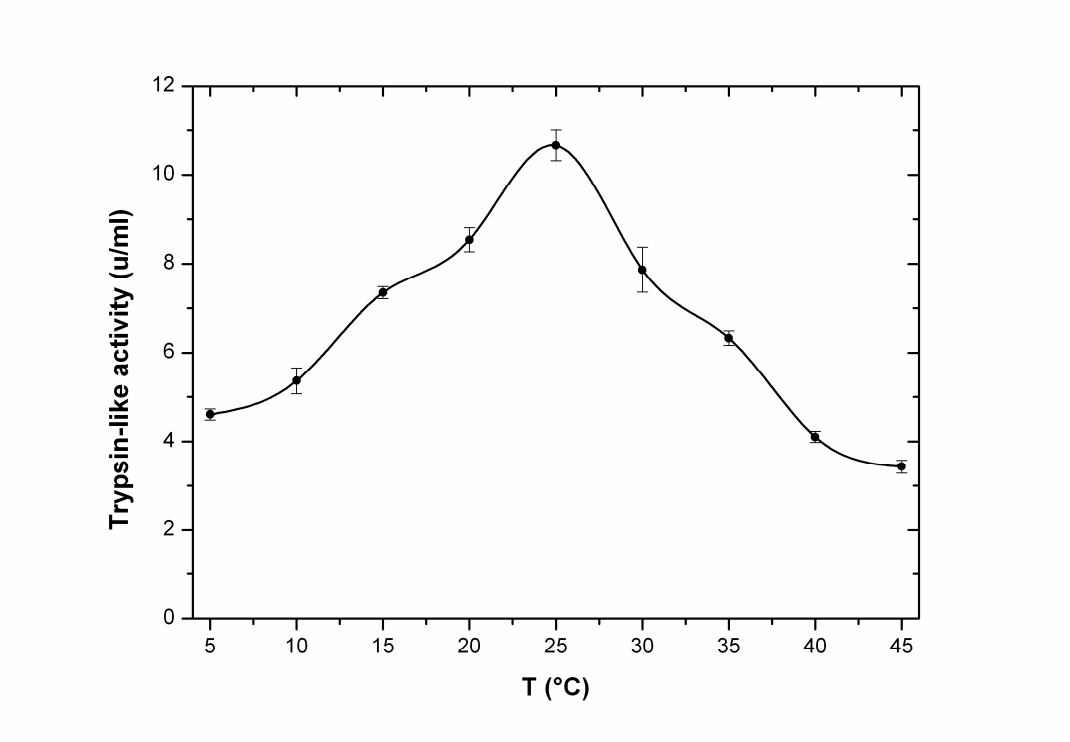

Trypsin-like activity (u/ml)
T (°C)
0
2
4
6
8
10
12
5
10
15
20
25
30
35
40
45

Fig. 2.