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Purification and characterization of a serine protease from *Cucumis trigonus* Roxburghi

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Abstract

Kachri fruit, *Cucumis trigonus* Roxburghi, contains high protease activity and has been used as meat tenderizer in the Indian subcontinent. A 67 kDa serine protease from Kachri fruit was purified by DEAE-Sepharose and CM-Sepharose chromatography, whose optimum activity was at pH 11 and 70 °C. Its activity was strongly inhibited by PMSF, but not by EDTA, pepstatin, or cysteine protease inhibitors. The substrate specificity of the purified protease towards synthetic peptides was comparable to cucumisin, the first characterized subtilisin class plant protease from the sarcocarp of melon fruit (*Cucumis melo*). These characteristics, along with the N-terminal amino acid sequence, indicated that the isolated protease from *Cucumis trigonus* Roxburghi is a cucumisin homologue, which belongs to the serine protease family.

Keywords: *Cucumis trigonus*; Cucurbitaceae; Plant protease; Serine protease; Kachri

1. Introduction

Proteolytic enzymes play significant roles in numerous cellular and extracellular processes. Several serine proteases have been isolated from distinct parts of plants ranging from seeds to latex and fruits. Plant serine proteases are involved in many physiological processes such as microsporogenesis, protein degradation, signal transduction and differentiation, and in hypersensitive response (Antao and Malcata, 2005). According to the classification of Barrett and coworkers, serine proteases are grouped into six classes, and the second largest class being the subtilisins (Barrett et al., 1998). All characterized members of the subtilisins are tripeptidylpeptidases or endopeptidases. Little direct evidence, however, is available about the enzymatic characteristics of plant subtilisin class proteases except for cucumisin.

Cucumisin from sarcocarp of melon fruit (*Cucumis melo*) was the first characterized plant subtilisin class protease (Kaneda and Tominaga, 1975; Yamagata et al., 1994). It comprised more than 10% of the total protein content in the fruit suggesting that it had an important role during fruit development (Yamagata et al., 1994). Subsequently, more cucumisin-like proteases were isolated from other plants, such as *Taraxacum officinale* Webb, *Euphorbia supine* and *Benincasa Hispida Var Ryukyu*, and characterized for their broad substrate specificity and optimum temperature and pH (Arima et al., 2000a,b,c; Tanaka et al., 2001; Yonezawa et al., 2000; Rudenskaya et al., 1998; Rudenskaya et al., 1995; Terp et al., 2000; Uchikoba et al., 1998). Although the subitilisin-like protease from tomato plants (PR-P69) had been related to the hypersensitive response (Tornero et al., 1997), the physiological role of cucumisin is largely unknown.

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Until now cysteine proteases are extensively used in the food and medicine industry. However, their activities are affected by air oxidation or metal ions, and by reducing or chelating agents. In contrast, various serine proteases including plant proteases have no requirement for any co-factors. Hence, it is highly desirable to identify and characterize novel serine proteases for the industrial utilization of plant proteases.

Traditionally, the Kachri fruit, *Cucumis trigonus* Roxburghi, has been used as meat tenderizer in the Indian subcontinent. The strong meat tenderizing activity of Kachri suggested the presence of high amount of proteases in the fruit. Therefore, we have chosen Kachri, a plant widely spread in Pakistan, for characterization of proteolytic enzymes. In this report, we have isolated and purified a novel serine protease from the Kachri fruit, and elucidated enzymatic characteristics as well as comparing its homology to other plant proteases.

2. Results and discussion

2.1. Purification of protease

The yield of each purification steps from 25 g fruit of *Cucumis trigonus* Roxburghi is presented in Table 1. About 1.6 mg of purified protein was purified with more than 95% homogeneity in SDS–PAGE (Fig. 1). The purification procedure consisted of two ion exchange chromatographic steps and ammonium sulfate precipitation. The purified protein appeared as a single subunit of 67 kDa, which is in agreement with other cucumisin-like serine proteases (Curroto et al., 1989; Kaneda et al., 1986; Kaneda and Tominaga, 1977; Rudenskaya et al., 1987; Santarius and Belitz, 1978; Yamagata et al., 1989). The content of the purified protease in the extract is estimated about 10% of total protein based on the total activity and amount of proteins in the extract. High content of the isolated protease in Kachri extract is comparable to that of cucumisin in melon fruit (Yamagata et al., 1994).

2.2. Sensitivity to various proteases inhibitors and substrate specificity

In order to identify the classes of isolated protease, the effects of different protease inhibitors have been examined. As shown in Fig. 2, typical inhibitors against cysteine, aspartic, and metallo-proteases had no effect on its activity. Nor did the reducing agent β-mercaptoethanol or the metal ions, Ca^{2+} or Mg^{2+}, indicating that the protease was not a cysteine, aspartic, or metallo-protease. The inhibitor of trypsin-like protease, ovomucoid, also had no effect. The strongest inhibition was observed with PMSF (phenylmethylsulfonylfluoride), a general inhibitor of serine proteases. No other tested inhibitors or compounds had any significant effect on the proteolytic activity. This inhibition profile classifies the isolated protease as a member of the subtilisin-like class of proteases. It is noticeable that the

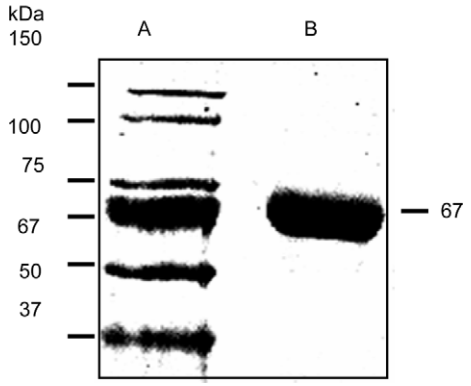

Fig. 1. SDS–PAGE of purified protease from *Cucumis trigonus* Roxburghi. (A) Protein markers and (B) purified protein from *Cucumis trigonus* Roxburghi.

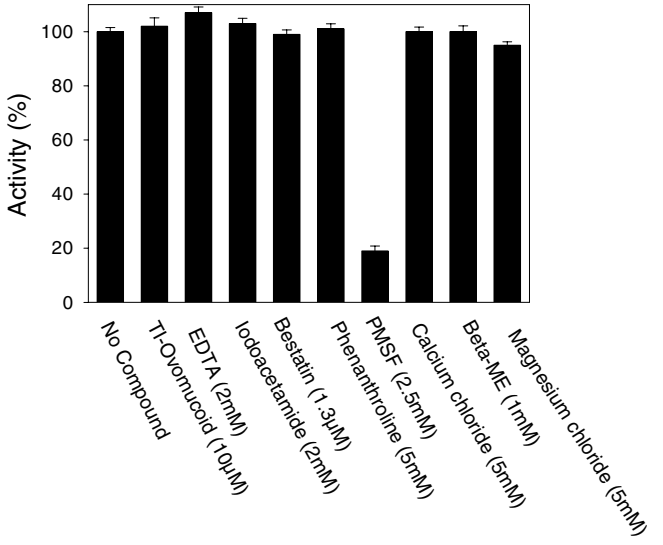

Fig. 2. Effects of various compounds on the protease activity. The enzyme was pre-incubated with various compounds of indicated concentration for 1 h, and the residual activities were measured using casein as substrate. These activities were compared with the activity of enzyme without any compound (100%).

Table 1
Purification of serine protease

Stage	Amount (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Cell extract	123	185,000	1500	1.0	100
DEAE-Sepharose	80	72,000	900	0.6	39
$(NH_4)_2SO_4$ precipitation	20	65,000	3155	2.0	35
CM-Sepharose	1.6	15,300	9742	6.5	8.2

isolated protease was not inhibited by ovomucoid, which is present in typical protein-rich food. The properties of this protease would, therefore, be advantageous for application in food industries.

The protease activity of the purified protein was tested against substrates that were commonly hydrolyzed by serine proteases (Table 2). The proteolytic activity with Suc-Ala-Ala-Pro-Leu-pNA was higher than those with Suc-Ala-Ala-Pro-Phe-pNA or Suc-Ala-Ala-Ala-pNA. Specially, the activity with Suc-Ala-Ala-Ala-pNA was only 2% compared to that of Suc-Ala-Ala-Pro-Leu-pNA as substrate. These results indicated that the purified protease preferred hydrophobic amino acid residues at the P1 position. The preference of hydrophobic residues at the P1 position of the isolated protease was comparable to the substrate specificities of chymotrypsin or elastase, which cleaves after large or small hydrophobic residues, respectively (Hartley, 1970; Kraut, 1977).

2.3. Optimum pH and pH dependent stability

The protease activity of the purified protein with casein as a substrate gradually increased until pH 12.0, and then decreased at higher pHs. Its activity at basic pH (pH 10–12) was 2-fold higher than that at neutral pH range (pH 6–9) (Fig. 3). The stability of the protease at different pH values was examined by measuring the remaining activity

Table 2
Substrate specificity

Substrate	Activity	Specific activity (μmol min^{-1} ml^{-1})	Relative activity[a] (%)
Suc-A-A-P-L-pNA	0.066	1.33	100
Suc-A-A-P-F-pNA	0.027	0.54	41
Suc-A-A-A-pNA	0.002	0.04	2.9

[a] The activity with Suc-A-A-P-L-pNA was defined as 100%.

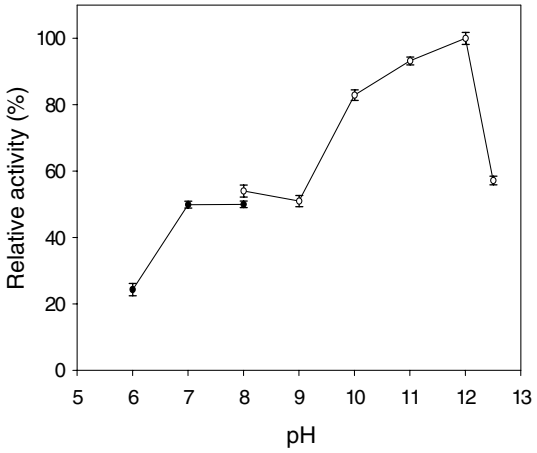

Fig. 3. Effects of pH on the activity of the protease. Enzyme activity was determined against casein at various range of pH 6.0–12.5. The buffers used for pH 6–8 were 100 mM sodium phosphate (filled circles), and pH 8–12.5 was 100 mM glycine-NaOH (open circles). Data are mean ± SD, $n = 3$.

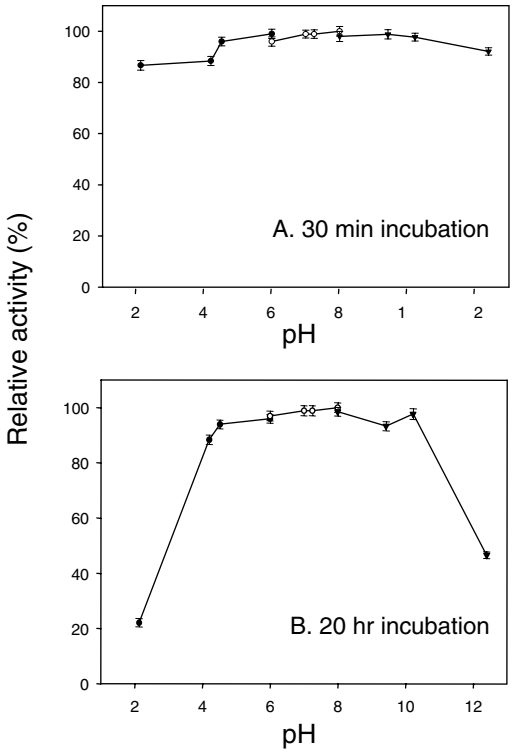

Fig. 4. Effects of pH on the stability of the protease. The enzyme activity was determined against casein at various pHs. The buffers used for pH 2–6 were 50 mM sodium acetate (filled circles), for pH 6–8 was 100 mM sodium phosphate (open circles) and pH 8–12.5 was 100 mM glycine-NaOH (triangles). The residual activity after incubation of 30 min (A) and 20 h (B) were measured. Data are mean ± SD, $n = 3$.

after incubation for 30 min or 20 h at various pHs ranging from 2.0 to 12.5. When the protease was incubated at different pHs for 30 min, there was no significant reduction of activity (Fig. 4A). The enzyme retained more than 80% of its initial activity after 20 h of incubation at 35 °C between pH 4.0 and 10.0. However, the activity rapidly decreased when incubated at pH 2 or 12 for 20 h (Fig. 4B) probably due to denaturation of the protein at extreme pH. The isolated protease was more stable at basic pH than other subtilisin class plant proteases, and its stability was comparable to those of cucumisin-like proteases from *Cucumis melo*, *Trichosantus kirrilowi* A, and *Trichosanthes bracteata* (Table 3).

2.4. Optimum temperature and thermal stability

The protease activity of the protein was examined using casein as a substrate at different temperatures ranging from 20 to 95 °C, and its activity increased as the reaction temperature increased from 20 to 70 °C. Indeed, its activity at 70 °C was 2.5- and 5-fold higher than that of the activities at 37 °C and 20 °C, respectively (Fig. 5A). The activity rapidly decreased as the reaction temperature increased higher than 80 °C due to thermal denaturation of the protein. The thermostability of the protease was examined by measuring the residual activity after incubation at different temperatures. When the protease was incubated between

Table 3
Characteristics of subtilisin class plant proteases

Source	M_W (kDa)	T_{op}[a] (°C)	pH_{op}[b]	Stability temperature	Stability pH	Substrate
Cucumis trigonus Roxburghi (this report)	67	70	11	to 70	4–10	Casein
Taraxacum officinale (Rudenskaya et al., 1998)	67	40	8.0	to 40	6–9	GAALP[c]
Heliantus annas (Uchikoba et al., 1990)	25	55	7.8	ND	4–10	ZAALP[d]
Machira pomifera (Rudenskaya et al., 1995)	65	58	8.5	ND	7–9	GAALP
Cucumis melo (Yamagata et al., 1989)	67	70	10.5	to 50	4–12	Casein
Cucurbita ficifolia (Curroto et al., 1989)	60	55	9.2	ND	8–11	Azocasein
Benincasa cerifera (Kaneda and Tominaga, 1977)	50	70	9.2	to 65	4.5–9.5	Casein
Benincasa hispida (Uchikoba et al., 1998)	67	60	10.0	to 60	7–11	Casein
Trichosantus cucumeroides (Kaneda et al., 1986)	50	70	7.3	to 60	7–11	Casein
Trichosantus kirrilowi A (Uchikoba et al., 1990)	50	70	10.0	to 50	4–12.5	Casein
Trichosanthes bracteata (Erlanger et al., 1961)	67	ND	11.0	ND	6–12	Casein
Euphorbia supine protease (Arima et al., 2000a)	80	ND	8.0	to 60	ND	Casein

ND, no data available.
[a] Optimum temperature.
[b] Optimum pH.
[c] Glp-Ala-Ala-Leu-pNA.
[d] Z-Ala-Ala-Leu-pNA.

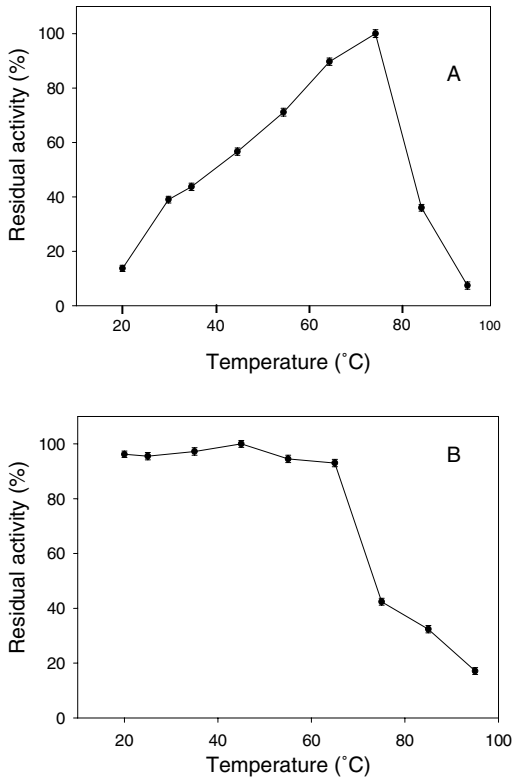

Fig. 5. Effects of temperature on the optimum activity and stability of the protease. The enzyme activity was measured at pH 7.4 for 20 min against casein as a substrate at various temperatures ranging from 20 to 95 °C. (A) The proteolytic activity of the purified protein was measured at different temperature. (B) The protease was incubated at various temperatures for 1 h, and the residual activity was measured. Data are mean ± SD, $n = 3$.

20 and 65 °C for 1 h, more than 90% activity was retained. However, the residual activity was sharply reduced when the protease was incubated at 75 °C or higher temperature (Fig. 5B). The 50% activity of protease remained around 70 °C, which was significantly higher as compared to the other subtilisin-class plant proteases, and the optimum temperature was comparable to *Cucumis melo*, *Benincasa cerifera*, *Trichosantus cucumeroides* Maxim, and *Trichosantus kirrilowi* A (Table 3).

2.5. Sequence analysis

The sequence of 10 amino acids at N-terminus of the purified protein was determined, and compared with other plant serine proteases (Fig. 6). Among the 10 residues, the sequence of first 7–8 residues of the isolated protease is identical to the cucumisin from *Cucumisin melo* (Yamagata et al., 1994), SCSI from soy bean (Povovic et al., 2002), white gourd protease (Uchikoba et al., 1998), and port C1 from soy bean (Povovic et al., 2002). The amino acid residues common to cucumisin-like protease, TTRSWDFL, were conserved in the purified protease. The N-terminal

	1 10
Cucumis trigonus Roxburghi	**TTRSWDFL**SG
Cucumisin melo L	**TTRSWDFL**GF
SCSI (soy bean) (D)	**TTRSWDFL**KS
White gourd	**TTRSWDFL**NF
Port C1 (soy bean)	**TTRSWDF**IGH
Tomato P69B	**TTRS**PTF**L**GL
Tomato P69A	**TT**HTSSF**L**GL
Bamboo protease	**TTR**TPSF**L**RL
Euphorbia supine protease B	**TTR**TPNF**L**GL
Alnus ag 12	**TT**HTPRF**L**SL
Lilly LIM9	**TT**HTPDY**L**GT
Arabidopsis	**TTR**TPLF**L**GL

Fig. 6. Comparison of the N-terminal amino acid sequences of various plant serine proteases. *Cucumis trigonus* Roxburghi (this report), *Cucumis melo* L (Yamagata et al., 1994), white gourd protease (Uchikoba et al., 1998), Port C1 (soy bean) and SCSI (soy bean) (D) (Povovic et al., 2002), tomato PR-P69 (Tornero et al., 1996) and tomato P69B (Tornero et al., 1997), bamboo serine protease (Arima et al., 2000b), *Euphorbia supine* protease B (Arima et al., 2000a), *Alnus* ag12 (Ribeiro et al., 1995), lily protease LIM 9 (Kobayashi et al., 1994) *Arabidopsis* protease (Yamagata et al., 2000). The bold print shows those amino acid residues identical in the sequences.

sequence of the protein also showed weak homology to the broad range of plant proteases such as P69A (Tornero et al., 1997) and P69B (Tornero et al., 1996) from pathogen induced tomato, bamboo protease (Arima et al., 2000b), *E. supine* protease (Arima et al., 2000a), alnus ag12 (Ribeiro et al., 1995), lily protease LIM9 (Kobayashi et al., 1994; Taylor et al., 1998) and an *Arabidopsis* protease (Yamagata et al., 2000).

3. Conclusions

The isolation and biochemical characterization of a plant serine protease from the fruit of *Cucumis trigonous* Roxburgi (Kachri) was presented. Molecular weight, enzymatic characteristics and strong homology of the N-terminal amino acid sequence to serine class plant proteases classified it as a member of cucumisin-like serine proteases. Its stability at basic pH and high temperature suggested that it may have a potential application in the food industry. The biochemical characterization of the isolated protease reported here is the first step to understand the function of this new cucumisin-like plant serine protease. Further studies will elucidate tissues localization of the protease and address the search for its physiological substrates.

4. Experimental

4.1. Materials

Kachri fruit *Cucumis rigonus* Roxburghi, was acquired in local market at Karachi, Pakistan. All the protease inhibitors, substrates including peptidyl-*p*NA (*para*-nitroanilide) and casein were from Sigma (USA). DEAE-Sepharose and CM-Sepharose were from Amersham Pharmacia (Sweden).

4.2. Protein purification

Enzyme purification was carried out at 4 °C. Frozen fruit (25 g) was homogenized with buffer A (50 mM sodium acetate, pH 5.0, 250 ml) in a Waring blender. The homogenate was centrifuged at 12,000 rpm for 1 h, and then the supernatant was loaded on the column of DEAE-Sepharose (1.8 × 20 cm) equilibrated with buffer A. The flow through fraction of DEAE-Sepharose column was subjected to 60% (w/v) ammonium sulfate precipitation, and the precipitated proteins were recovered by centrifugation at 15,000*g* for 30 min. The resulted pellet was resuspended, dialyzed with buffer A, and applied on to a CM-Sepharose column (1.5 × 12 cm) equilibrated with buffer A. Proteins were eluted from the column with a linear gradient of 0–0.16 M NaCl in buffer A. Proteolytic activity in each fraction was measured using casein as a substrate. The active fractions were collected and analyzed for purity. SDS–PAGE was performed as previously described (Laemmli, 1970) with 12.5% acrylamide in separating gel. The molecular mass was estimated by SDS–PAGE using the gel pro analyzer software 3.1 (Media Cybernetics, USA).

4.3. Enzyme assay

Proteolytic activity was measured with casein as a substrate as described previously (Kaneda and Uchikoba, 1994). Briefly, enzyme solution (100 μl) was added to 1 ml of 2% (w/v) casein in 100 mM sodium phosphate, pH 7.4. After incubation at 35 °C for 20 min, the reaction was stopped by the addition of 5% trichloroacetic acid (TCA, 3 ml). After removing the precipitate by filtration through Whatman #1 filter paper, the absorbance of the filtrate at 280 nm was measured using a BioPhotometer (Eppendorf, Germany). A unit of protease activity was defined as the activity resulting 0.001 absorbance unit of increase at 280 nm per min under the above conditions.

Proteolytic activity was also examined by measuring the hydrolysis of peptidyl-*p*NAs. The enzyme solution (0.2 ml) was added to 0.6 ml of 0.2 M Tris–HCl, pH 7.5, containing 10 μl of 10 mM peptidyl-*p*NA in DMSO. The rate of enzymatic hydrolysis for peptidyl-*p*NA substrates was measured using a lambda 18 spectrophotometer (Perkin–Elmer, Jügesheim, Germany). An extinction coefficient of 8800 $M^{-1}\,cm^{-1}$ at 410 nm was used for the yield of the hydrolysis product, *p*-nitroaniline (Erlanger et al., 1961). A unit (u) of the enzyme activity was defined as the amount of the enzyme required releasing 1 μM of product per min.

4.4. Characterization of enzyme activity

The influence of different kinds of protease inhibitors on enzyme activity was tested by incubating the compounds at the indicated concentration in the assay buffer (100 mM sodium phosphate, pH 7.4) for 1 h at 35 °C prior to the addition of the substrate. Enzyme activities after the treatments were then measured by adding 2% casein solution as describe above.

The pH stability of the purified protease was determined by measuring the remaining casein hydrolyzing activity after incubation for 30 min or 20 h at 35 °C in different buffers (50 mM sodium acetate buffer between pH 2 and 6, 100 mM sodium phosphate buffer between pH 6.0 and 8.0 and 0.1 M glycine between pH 8 and 12.5). The temperature stability was determined by measuring the remaining activity after the incubation of enzyme solution in 100 mM sodium phosphate, pH 7.4 for 1 h at different temperatures. The optimum pH was determined by measuring the casein hydrolyzing activity at 35 °C for 20 min in different pH range (pH 6–12.5). The buffers used were 100 mM sodium phosphate (pH 6.0–8.0) and 0.1 M glycine (pH 8–12.5 adjusted with NaOH). The optimum temperature for the enzyme activity was determined by measuring the activity at 20–95 °C in 100 mM sodium phosphate, pH 7.4.

4.5. Determination of N-terminus amino acid sequence

After 12.5% SDS–PAGE of purified protein sample, the protein was transferred to PVDF membrane (Pierce Inc, USA), and stained with ponceau-s solution (Sigma). N-terminal amino acid sequence was determined by automated Edman degradation with an Applied Biosystems 477 A protein sequencer (Foster City, CA, USA). The phenylthiohydantoin derivatives were identified with an Applied Biosystems 120A analyzer (Foster City, CA, USA).

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