Mem. Fac. Agr. Kagoshima Univ., **59**, 37-42, 2009

Keratinolytic Properties of PrP^{Sc}-degrading Enzyme E77.

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Received for Publication, September 10, 2008

SUMMARY

It is suggested that the keratinolytic activity of proteases are associated with the degrading activity of the abnormal type prion protein (PrP^{Sc}). We tried to analyze the keratinolytic properties of the PrP^{Sc}-degrading enzyme E77. E77 showed a much greater keratinolytic activity than proteinase K and subtilisin. The maximum activity of keratin was observed at a pH value of 12.0 at 65°C. E77 also showed a pH-independent adsorbability for keratin. These profiles of E77 were the same as the PrP^{Sc}-degrading enzyme NAPase, except for high alkaline conditions. E77 and NAPase showed lower collagenolytic and elastinolytic specificities.

Key words: Prion; keratinase; protease

INTRODUCION

Prion diseases are characterized by the conversion of the normal cellular form of the prion protein (PrP^{C}) into an insoluble, protease-resistant abnormal form (PrP^{Sc}) [1, 2]. There have been some reports of PrP^{Sc}-degrading enzymes, but the keratinase from *Bacillus licheniformis* [3] and the subtilisin-enzyme Properase [4] need additional chemical and physical treatments for the degradation of PrP^{Sc}. Only an alkaline protease E77 from *Streptomyces* sp. and NAPase from *Nocardiopsis* sp. TOA-1 have been reported to be able to degrade PrP^{Sc} without further treatment [5, 6].

It has been reported that the specificity toward keratin is an important factor in the degradation of PrP^{Sc} by proteases [3, 7, 8]. In a previous paper, we also reported that NAPase possessing a keratin-degrading ability was able to degrade PrP^{Sc} [9, 10]. Nevertheless, the PrP^{Sc}-degrading protease E77 has not been investigated in terms of its keratin degrading properties. In this paper we describe the keratinolytic properties of PrP^{Sc}-hydrolysing E77.

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MATERIALS AND METHODS

Preparations of the enzyme and protein content

The alkaline protease E77 from *Streptomyces* strain 99-GP-2D-5 and NAPase from *Nocardipsis* sp. TOA-1 were purified utilizing the same process as described in a previous paper [5, 9]. Subtilisin Carlsberg and proteinase K (Sigma-Aldrich) were purified by cation-exchange and gel-filtration chromatographies, respectively. The protein content was determined by the Lowry method [11] using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophresis (SDS-PAGE) was prepared as described by Laemmli [12].

Protease activity assay

The protease activity was measured using the Anson-Hagiwara's method [13]. 1.0 ml of 0.6% casein in a 50 mM glycine-NaCl-NaOH buffer (pH 10.5) and 0.2 ml of enzyme solution were incubated at 30°C. After incubation for 30 min, the reaction was brought to an end by the addition of 2.5 ml of trichloroacetic acid (0.11 M). The mixture was further incubated at 30°C for 30 min, and filtered through a Toyo filter paper (No. 5C). The free amino acids in the filtrate were measured with a Folin-phenol reagent.

Keratinase activity assay

Forty μg of E77, subtilisin or proteinase K and 60 mg of keratin powder (Tokyo Kasei) were incubated with shaking in a 3 ml glycine-NaCl-NaOH buffer (pH 10.5) and a 50 mM KCl-NaOH buffer (pH 12.5) at 30°C for 2 hours. The reaction was brought to an end by adding a 2.5 ml of 0.11 M trichloroacetic acid solution. The mixture was further incubated at 30°C for 30 min and filtered. The liberated peptides in the filtrate were measured photometrically at 280 nm. One unit of keratinolytic activity was defined as the amount of enzyme that released 1 μg of tyrosine per hour. The optimal pH and temperature were measured at pH values ranging from 7.0 to 12.5 and at temperatures ranging from 30 to 70°C.

Keratin absorbability assay

Fifteen μg of E77, subtilisin or proteinase K and 50 mg of keratin powder were incubated at 4°C in various 1 ml buffers: 50 mM Tris-HCl (pH 8.0), 50mM glycine-NaCl-NaOH (pH 9.0 - 12.0). After 30-min incubation, the reaction mixture was centrifuged (10,000Xg for 5 min) and the remaining caseinolytic activity of supernatant was measured.

Collagenase and elastase activity assay

Forty μg of E77, NAPase, subtilisin or proteinase K and 60 mg of collagen or elastin (Sigma-Aldrich) were incubated with shaking in a 3 ml Tris-HCl buffer (pH 8.0) and a 50 mM glycine-NaCl-KCl-NaOH buffer (pH 10.5) at 30°C for 1 hour. The reaction was brought to an end by adding 5μl of 10 M acetic acid. After centrifugation at 4°C and 10,000Xg for 10 min, 0.5 ml of each reaction mixture was added to a 0.5 ml of 0.2 M sodium acetate buffer (pH 5.0). After this, 1 ml of a ninhydrin reagent (Sigma-Aldrich) was added; the free amino groups were measured according to the manufacturer's instructions. One unit of the activity was defined as the amount of enzyme that released 1 μg of glycine per hour.

RESULTS AND DISCUSSON

Properties of the keratin hydrolysis

Fig. 1 shows the time courses of the keratin hydrolysis by E77. Comparing these results with our previous paper, E77 exhibited a comparable hydrolyzing-ability toward keratin with NAPase [9, 10]. The keratinolytic activity of E77 at pH 10.5 was about 5- and 3-fold increase on the results obtained with subtilisin and proteinase K, respectively. The specific activity at a pH value of 10.5 (3,700 unit/mg) was slightly higher than NAPase [9]. The optimal pH and temperature of E77 were 12.0 and 65°C, respectively (Fig. 2). E77 showed a lower keratinolytic activity than NAPase under high alkaline conditions because of the difference in pH stability (data not shown).

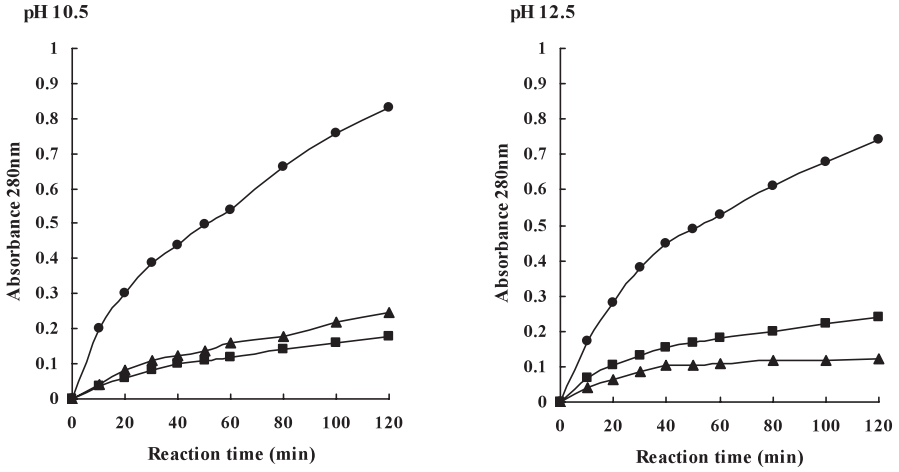

Fig. 1. Hydrolysis of keratin by E77, subtilisin and proteinase K. The reaction was carried out at 30°C. Symbols: ●, E77; ■, subtilisin; ▲, proteinase K.

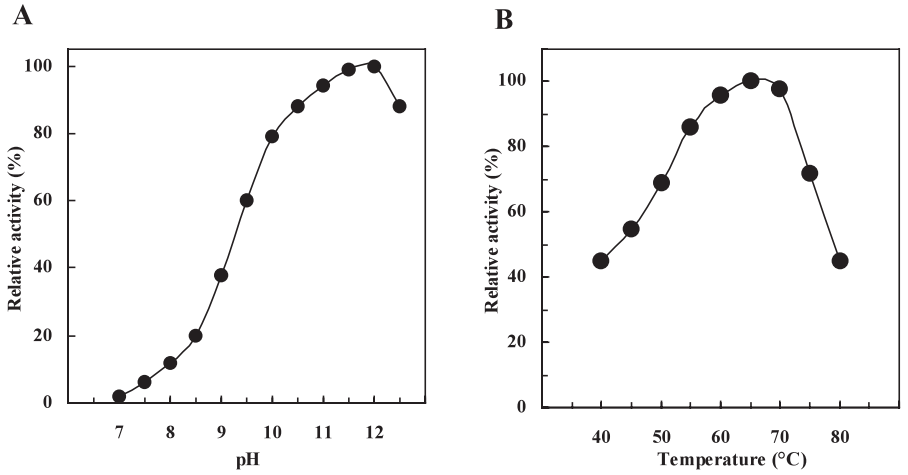

Fig. 2 A, B. Effects on pH and temperature of the keratinolytic activity of E77. (A) Effects on pH of the keratinolytic activity. The reaction was carried out at 30°C. (B) Effects on temperature of the keratinolytic activity. The reaction was carried out at pH 10.5.

Properties of keratin absorption

The absorbability of E77 was observed at pH values ranging from 8.0 to 12.0 (Fig. 3). E77 showed more than 70% of the adsorption rate for keratin, which was pH-independent. Comparing these results with our previous paper, E77 exhibited a comparable adsorbability for keratin with NAPase [10]. E77 and NAPase possess a high absorbability for insoluble substrates in agreement with other insoluble substrate-hydrolyzing enzymes [14-17].

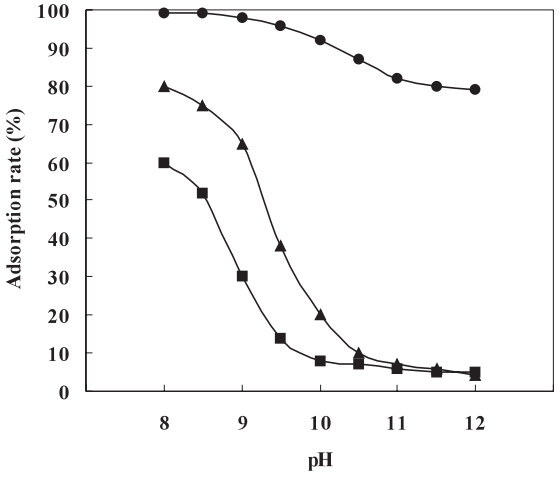

Fig. 3. Effects on pH of the keratin adsorbability of E77, subtilisin and proteinase K. Symbols: ●, E77; ■, subtilisin; ▲, proteinase K.

Table 1. Enzyme activities with collagen and elastin.

Enzyme	Specific activities (unit/mg):			
	pH 8.0		pH 10.5	
	collagen	elastin	collagen	elastin
E77	1290	1040	1940	1400
NAPase	1270	950	1670	1100
Proteinase K	1670	1230	2440	1180
Subtilisin	1850	1660	2560	1270

Properties of the insoluble substrates hydrolysis

Table 1 shows the hydrolyzing activities of E77, NAPase, proteinase K and subtilisin with collagen and elastin. E77 and NAPase had a lower ability to hydrolyze proteins than proteinase K and subtilisin. The relative activity (specific activity of keratin versus collagen) at a pH value of 10.5 was about a 5 fold increase in the results obtained from proteinase K and subtilisin.

We came to the conclusion that the keratin-hydrolyzing and adsorbable abilities of these enzymes are closely related to the degrading ability of PrP^{Sc}. Further studies using mutant E77 and NAPase are currently underway to reveal insights into the molecular mechanism of keratin and PrP^{Sc}-degradation.

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異常プリオン分解酵素E77のケラチン分解特性

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要　約

プロテアーゼのケラチン分解能と異常プリオン分解能には相関があるのではないかと推察されている。そこで我々は，先に報告した異常プリオンタンパク質分解酵素E77のケラチン分解特性について解析した。E77の比活性は3670unit/mgであり，proteinaseKおよびsubtilisinと比較し，ケラチンを強力に分解することが明らかになった。E77のケラチン分解能の至適pHは12.0，至適温度は70℃であった。また，pH非依存型の高い吸着能を示すなど，高アルカリ領域以外では，先に報告した異常プリオン分解酵素NAPaseとほぼ同様の特性を示した。一方，E77のコラーゲンおよびエラスチンに対する特異性はNAPaseと同様低かった。

キーワード：異常プリオン，ケラチナーゼ，プロテアーゼ

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