Note

Further Stabilization of Earthworm Serine Protease by Chemical Modification and Immobilization

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Earthworm serine protease is more stable and is less affected by organic solvents and detergent than other proteases. However, it is inactivated, probably by autolysis, at 60°C or above under alkaline conditions. Further stabilization was managed by chemical modification of the enzyme with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and phenylglyoxal to protect the activity from the autolytic inactivation. Stabilization was possible also under acidic conditions, in which the stability of the enzyme was rather low, by immobilization with folded sheet mesoporous material. Thus, further stabilization of the enzyme has been achieved by chemical modification or immobilization.

Key words: serine protease; earthworm; stabilization; chemical modification; immobilization

Earthworms secrete serine proteases that degrade a wide variety of proteins such as fibrin, collagen, and elastin, and the lyophilized powder of the earthworms have long been used as a drug for various diseases in Chinese medicine under the name 'jiryu'.¹⁻³ The therapeutic effects of these fibrinolytic proteases from earthworms have been reported.⁴⁻⁶ We have characterized and cloned alkaline serine proteases with fibrinolytic activity from the earthworm Lumbricus rubellus, and showed that the proteases are composed of six isozymes (isozymes A, B, C, D, E, and F, formerly named F-III-2, F-III-1, F-II, F-I-2, F-I-1, and F-I-0, respectively), derived from different genes. On the basis of results of their cleavage specificity against peptide substrates, iso-zymes A, B, D, E, and F had both trypsin- and chymotrypsin-like activities, but C also acted like an elastase. The enzymes retain their activity for years at room temperature and are little affected by various organic solvents; however, inactivation occurs apparently by autolysis (auto-digestion) at 60°C or above under alkaline conditions.⁷⁻¹¹ We describe here further stabilization of the earthworm protease by chemical modification under alkaline conditions to protect the activity from the autolytic inactivation, and also stabilization by immobilization under acidic conditions, in which the stability of the enzyme was rather low.

The earthworm protease isozyme A had the highest activity of the six isozymes. Therefore, the enzyme, which was completely purified as we previously described,⁷,¹⁰ was used in the following experiments. Trypsin (porcine, type IX) was purchased from Sigma Chemical Co., USA. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and phenylglyoxal (PGO) were from Nacalai Tesque, Japan. The folded sheet mesoporous material (FSM) 16/52 was kindly provided by Dr. H. Takahashi, Toyota Central R&D Laboratories, Inc., Aichi, Japan. The other reagents were of analytical grade. Enzyme activity was assayed with chromogenic substrates such as H-D-Phe-Pip-Arg-pNA for isozyme A and Bz-L-Arg-pNA for trypsin.⁵,¹⁰ Protein was measured by the method of Lowry et al.³

Isozyme A was more stable than trypsin and chymotrypsin. The enzyme had a strong tolerance to organic solvents (25% v/v) for at least more than 100 days at room temperature, even towards toluene and hexane as described previously,¹⁰ and retained activity for at least 20 days in 10 mM Tris-HCl buffer (pH 8.0) separately containing SDS (1%), Triton X-100 (1%), and NaCl (20%), compared with the complete inactivation of trypsin as a control. The enzyme kept at least 80% of its activity for at least 5 years at room temperature.¹⁰ Moreover, it maintained almost all of its activity for more than 10 days up to 55°C under alkaline conditions (pH 8.0) (Fig. 1), although the

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stability of the enzyme was rather low at pH 6.0, like other alkaline serine proteases. Such stability and tolerance like those described above were found in other isoizymes, isozyme B and C.

A rapid decrease in the activity of isozyme A occurred above 60°C upon heating for 30 min under alkaline conditions (pH 9.0) (Fig. 2(A)). Inactivation was seemed to be accompanied by autolysis, because the temperature (at 60°C), at which the inactivation occurred by heating for 30 min coincided with that on the degradation of the enzyme protein into two peptide fragments by SDS-PAGE (Fig. 2(B)). Heating for longer than 30 min at a higher temperature caused the appearance of many smaller peptides from the native polypeptide. The autolytic cleavage of isozyme A occurred at the site of Arg(144)-Tyr(145) of the polypeptide. The enzyme has no Arg or Lys residues in the sequence comprising Asn-131 to Asp-141 corresponding to the autolysis loop (Asn-143 to Asp-153) of bovine trypsin but has Arg-144, which triggers the autolysis of the enzyme. These results provide information concerning the higher stability of the enzyme.

We tried further stabilization of isozyme A by chemical modification (cross-linkage) with EDC as previously described. Compared with the native enzyme, the EDC-modified enzyme, dissolved in 50 mm Tris-HCl buffer (pH 9.0), maintained its activity against heating at 65°C for 30 min and for around 10 h at 60°C (Fig. 3). The modified enzyme was not degraded into peptide fragments with heat treatment at least for 30 min at 60°C on SDS-PAGE. The original specific activity of the enzyme for the chromogenic substrate was 150 units/mg protein, but, the activity of the enzyme decreased by two thirds after the modification. Similar to the results of the EDC modification, further stabilization of the enzyme was also possible by chemical modification with PGO, when 1 mg of the enzyme was incubated at 30°C for 1 h with 0.1 mm of PGO in 5 ml of 50 mm potassium phosphate buffer (pH 8.0) containing 1 mm EDTA and 1 mm dithiothreitol by previously described methods. These stabilizations against heating (up to 65°C) via the chemical modification with EDC and PGO seemed to be due to blocking of the Arg residues including the R144-site by chemical modification for the protection of the activity from auto-lytic degradation.

Fig. 1. Stability of the Earthworm Protease (Isozyme A) under Both Alkaline and Acidic Conditions.
Isozyme A (1 μg) was incubated in 10 ml Tris-HCl buffer (pH 8.0) or 10 mm potassium phosphate buffer (pH 6.0) for various times at 55°C. The remaining activity was assayed in 1 ml of 100 mm Tris-HCl buffer (pH 9.0) as described in the text.

Fig. 2. Inactivation Curve of the Enzyme by Heating and the Degradation Pattern of the Enzyme Protein into the Peptide Fragments by Autolysis.
(A): Isozyme A (1 μg) was incubated in 1 ml of 50 mm Tris-HCl buffer (pH 9.0) at various temperatures for 30 min. The remaining activity was assayed in the buffer as described in the text. (B): The degradation pattern of the enzyme protein was analyzed by SDS-PAGE (12.5%) before (1) and after (2) heat treatment for 30 min at 60°C in the buffer. M, standard protein markers (Pharmacia Biotech). Arrow shows two peptide fragments produced by autolysis.
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Fig. 3. Heat Stability of the EDC-Modified Enzyme and the Native Enzyme.
Iszyme A (1 mg) was modified in 1 ml of 50 mM sodium acetate buffer (pH 4.0) containing 10 mM EDC and 2 mM N-hydroxysuccinimide for 2 h at room temperature by the procedures described in the text. The remaining activities of the modified (3 μg) (●) and the native (1 μg) (▲) enzymes were assayed in 1 ml of 50 mM Tris-HCl buffer (pH 9.0) after heat treatment at various temperatures for 30 min in the buffer (A) and for various times at 60°C (B).

ic inactivation.9,11)

As shown in Fig. 4, it was possible to stabilize iszyme A at 30°C by immobilization with FSM18 under acidic conditions (10 mM sodium acetate buffer, pH 5.0), in which the original stability of the enzyme was rather low (see Fig. 1), although the specific activity (150 units/mg) of the enzyme decreased by four fifths due to the immobilization.

The chemical modification with both 2,3-butanedione9 and glutaraldehyde,20 and the immobilization with both epoxy-activated silica11 and CNBr-activated Sepharose 4B20 were not effective for further stabilization of the enzyme under either alkaline or acidic conditions.

Thus, we have achieved further stabilization of the earthworm protease by chemical modification with EDC and PGO under alkaline conditions, probably by protecting the activity from autolytic inactivation, and also the stabilization by immobilization with FSM under acidic conditions, in which original sta-
bility of the enzyme was rather low. This protease, which has a high stability and wide-ranging substrate specificity would be useful for therapeutics, for the degradation of organic waste products, and for the synthesis of useful compounds.

References