Preparation of Milk-clotting Enzyme from Basidiomycete

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In the selection of rennet substitutes, it is well known that the ratio of milk-clotting activity to proteolytic activity (MCA/PA) is an important index and higher MCA/PA ratio is desirable. Thus, a simple preparation method was investigated for the elimination of proteinase having high proteolytic activity from the rennet-like enzyme of *Irpex lacteus*. At the treatment of pH 6.5, 35°C for 20 min, the proteinase other than the milk-clotting enzyme was inactivated almost completely and the MCA/PA ratio increased approximately 1.8-fold over that of the crude preparation. This value closely equaled that of the milk-clotting enzyme of *Irpex lacteus*, purified by an affinity chromatography. It is concluded that this advantageous treatment can be easily applied commercially for cheese manufacture, because it is very simple and cheap.

Cultured filtrate of *Irpex lacteus*, a basidiomycete, contains milk-clotting enzyme and proteinase with high proteolytic activity. Although both enzymes belong to the aspartic proteinase which has optimum pH at the acidic region, the milk-clotting enzyme is inhibited completely by pepstatin^{1,2)}, a specific aspartic proteinase inhibitor, at a concentration of 10^{-5} M³⁾, whereas the co-existing proteinase is not affected by pepstatin at all even at a concentration of 10^{-3} M⁴⁾. We have already reported¹⁾ that the milk-clotting enzyme is separated from the contaminated proteinase by

affinity chromatography having a pepstatin derivative as a ligand. We have also made a Cheddar cheese with the milk-clotting enzyme fraction of *Irpex lacteus* purified by affinity chromatography and have shown a possibility of using the milk-clotting enzyme as a calf rennet substitute by comparing it with the cheese made with calf rennet⁵⁾. However, it is too expensive to apply affinity chromatography to get the milk-clotting enzyme fraction on a large scale.

In this paper, we report a simple method for inactivating the proteinase having high proteolytic activity to get the milk-clotting enzyme.

1. Materials and Methods

Materials

Calf rennet powder was purchased from Chr. Hansen Lab. Denmark and *Mucor pusillus* rennet was obtained from Meito Sangyo Co. Ltd. Japan. Pepstatin was obtained from Protein Research Foundation of Japan, hemoglobin was purchased from Sigma Chemical Co., and casein was from Merck. SVP (distiller's soluble, Sun Grain) was obtained from Suntory, Ebios was from Asahi Breweries Co. Ltd. and Nonion E-230 was from Nihon Yushi Co. Ltd.

Enzyme assays

Milk-clotting and proteolytic activities were measured as described in our previous paper³⁾.

Estimation of pepstatin inhibition

To 0.99 ml of enzyme solution, 10 μ l of pepstatin solution (10^{-2} M/DMF) was added and the mixture was immersed in an ice bath for 15 min. Then the remaining activity was determined with hemoglobin as the substrate at pH 3.0 and 35°C for 30 min. The pepstatin inhibition was calculated as follows,

$$\text{pepstatin inhibition (\%)} = (A-B)/A \times 100$$

A : proteolytic activity without pepstatin

B : proteolytic activity with pepstatin

Media and culture conditions

The seed medium contained 4% sucrose, 3% SVP (distiller's soluble), 0.3% yeast extract, 0.5% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and the pH of the medium was adjusted to 4.0. The production medium contained 6% sucrose, 3% SVP, 0.6% Ebios, 0.6% $\text{NH}_4\text{H}_2\text{PO}_4$, 0.05%

MgSO₄ · 7 H₂O, 0.1% lactic acid, 1 ppm calcium pantothenate, 10 ppm choline chloride, 0.2 % Nonion E-230, and the pH of the medium was adjusted to 3.8. Seed culture was carried out in 500 ml flasks containing 60 ml of seed medium at 30°C for 4~5 days at 125 opm. Enzyme production was conducted in a 5 liter jar fermentor containing 2 liters of production medium at 28°C. Agitation and aeration was kept at 750 rpm and 0.5 vvm, respectively.

2. Results and Discussion

Production of the enzyme began after 50 h cultivation, and the enzyme activity reached maximum at 120 h fermentation. Then the cultured broth was centrifuged at 9 000 × g for 15 min, and ammonium sulfate was added to the supernatant to bring 80% saturation. The resultant precipitate was collected by centrifugation and dissolved in small quantities of water. The solution was dialyzed against 0.01 M acetate, pH 5.5, and used as crude enzyme solution during the subsequent studies.

The crude enzyme solution contains milk-clotting enzyme and pepstatin-insensitive aspartic proteinase having high proteolytic activity. It is possible to determine the content of the milk-clotting enzyme in the crude enzyme preparation, because the milk-clotting enzyme is inhibited by pepstatin at the concentration

of 10⁻⁴M completely, whereas the proteinase is not affected at all at the same concentration⁴⁾. Thus, the remaining proteolytic activity at pH 3.0 after the treatment with pepstatin indicates the activity of pepstatin-insensitive aspartic proteinase.

Analysis of this pepstatin-insensitive aspartic proteinase revealed that the ratio of milk-clotting activity to proteolytic activity on casein at pH 6.0 was one-fourth of that of the milk-clotting enzyme and one-second of that of the crude enzyme. To determine the optimum condition for eliminating the pepstatin-insensitive aspartic proteinase, the stability of the milk-clotting enzyme and of the proteinase was investigated. After the treatment at conditions of 30°C for 1 h at various pHs, the milk-clotting enzyme was stable between pH 3 to 6, whereas the proteinase was stable between pH 3 to 5. It is obvious that the proteinase is relatively less stable at neutral pH region than the milk-clotting enzyme.

To select the condition suitable for inactivating the proteinase in the crude preparation; pH, temperature and incubation time was changed, and then the remaining proteolytic activity at pH 3 and pepstatin inhibition were measured. If the proteinase is inactivated completely, the pepstatin inhibition must in-

Fig. 1 Effects of heat treatment on pepstatin inhibition and the stability of the crude enzyme

The experimental conditions were the following: A, at various pHs and 35°C for 20 min; B, at various temperatures and pH 6.5 for 20 min; C, at 35°C and pH 6.5 for various time intervals. ○, pepstatin inhibition; ●, remaining activity.

Table 1 Effect of heat treatment on milk-clotting activity to proteolytic activity (MCA/PA) ratio and pepstatin inhibition of the crude enzyme

	Pepstatin inhibition(%)*	MCA (su/ml)	PA(unit)**	MCA/PA
Heat treatment***	99.4	1 791	1.9	943
Control****	77.0	1 832	3.5	523

*The remaining activity was determined using 1.2% hemoglobin (pH 3.0) as substrate at 35°C for 30 min after the treatment with 10^{-4} M pepstatin at 0°C for 15 min.

**The proteolytic activity was determined using 1.2% casein (pH 6.0) as substrate at 35°C for 30 min.

*** Heat treatment of pH 6.5 and 30°C for 20 min

**** Treatment of pH 6.5 and 0°C for 20 min

crease to 100% and the proteolytic activity at pH 3 remains at about 80% of the control. By the treatment of 35°C for 20 min, pepstatin inhibition reached the level of nearly 100% from pH 6.3 to 6.7 (Fig. 1-A). But the optimum pH for inactivating the proteinase was found to be from 6.3 to 6.5, because the remaining activity by the treatment at pH 6.7 was less than those at pH 6.3 and pH 6.5 treatments. When the crude enzyme was incubated at pH 6.5 for 20 min at various temperatures, almost complete pepstatin inhibition was observed above 35°C (Fig. 1-B). In the incubation at 40°C, the proteolytic activity decreased to less than 70% of the original activity, indicating that not only proteinase but also milk-clotting enzyme was inactivated above 40°C. When the crude enzyme was incubated at pH 6.5 and 35°C, the pepstatin inhibition rate increased to nearly 100% at 20 min incubation (Fig. 1-C). At this stage, the proteolytic activity was shown to be 80% of the control, after that the proteolytic activity decreased further with prolonged incubation time. Thus, the optimum condition for treating the *Irpex* crude enzyme was determined to be at pH 6.5 and 35°C for 20 min.

After incubating the crude enzyme with the condition suitable for the elimination of proteinase, i.e. incubation at pH 6.5 and 35°C for 20 min, the residual activity with pepstatin treatment was reduced from 23% to 1% of the original activity (Table I). This fact indicates that the proteinase existing in the crude preparation is inactivated almost completely by the

heat treatment. In addition, the MCA/PA ratio increased 1.8-fold by this treatment over the crude preparation. This value closely equals that of the milk-clotting enzyme fraction of *Irpex lacteus* obtained from affinity chromatography³⁾. The decrease of the milk-clotting activity was less than 3% of the original activity, indicating that the milk-clotting enzyme was still fully active after the treatment. It is suggested that this advantageous treatment can be easily scaled up commercially for cheese manufacture, because it is really simple and cheap. Evaluation of heat-treated enzyme by actual cheese making test will be published elsewhere.

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担子菌凝乳酵素の調製

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仔牛レンネット代替品を選択する際、タンパク分解活性に対する凝乳活性の比 (MCA/PA) は重要な指標で

あり、より高い MCA/PA 比が望まれる。そこで、*Irpex lacteus* のレンネット様酵素から、高いタンパク分解力をもつプロテアーゼを除去するための簡単な凝乳酵素調製法を研究した。pH 6.5, 35°C, 20 min の処理で、凝乳酵素以外のプロテアーゼはほぼ完全に失活し、MCA/PA 比は粗酵素標品の約 1.8 倍に上昇した。この値は、アフィニティークロマトグラフィーで精製された *Irpex lacteus* の凝乳酵素の値とよく似ていた。この処理法は簡単で安価であり、チーズ製造工業に容易に応用できると結論される。