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# STUDIES ON THE IMMOBILIZED CHYMOSIN PART III

## Preparation and Properties of Immobilized Chymosin using Photo-crosslinkable Resin Prepolymers

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### Abstract

Immobilized chymosin was prepared by entrapment with photo-crosslinkable resin prepolymers. The relative activity of the immobilized chymosin was affected by illumination time and chymosin concentration. Immobilized chymosin showed higher activity than native chymosin at high temperature. Native chymosin was unstable at pH 4.1, but immobilized chymosin was remarkably stable at pH below 5.4. Heat stability was increased by immobilization. Batch-wise and continuous enzymatic treatment was achieved by the immobilized chymosin.

### Introduction

Immobilized enzymes in a relatively new field has been extensively studied and has been reviewed by several authors<sup>1-3)</sup>.

Immobilized chymosin can offer certain advantages over soluble chymosin in the study of the enzymatic coagulation of milk, and in their potential application to cheese manufacture. Use of immobilized chymosin would allow the reuse of the chymosin and alleviate the shortage of commercial rennet. Moreover it may be possible to reveal the mode of action of chymosin on milk proteins and the casein micelle structure.

Enzymatic milk coagulation can be divided into two phases: the primary (enzymatic) phase, where the phenylalanyl — methionine bond in k-casein is cleaved, and the secondary (nonenzymatic) phase in which there is a subsequent physical aggregation of micelles to form a coagulum. The greater sensitivity of the secondary phase to factors such as pH and temperature

permits separation of the two phases. The basis for the application of immobilized chymosin in milk coagulation is to take advantage of the above characteristics. That is to say, the immobilized chymosin retains sufficient activity at the lower temperature and higher pH of milk to complete the primary phase; subsequent clotting can be accomplished by warming the milk or lowering its pH after milk is removed from the immobilized chymosin.

In this paper, we prepared immobilized chymosin using photo-crosslinkable resin prepolymers and studied its characteristics and the possibility of using it for a continuous milk-clotting system.

## Materials and Methods

### I. Purification of chymosin

Chymosin was purified from commercial cheesemaking rennet powder (Chr. Hansen, Reading, Berks) according to DEAE-cellulose chromatography, as described by YOSHINO *et al.*<sup>4)</sup>

### II. Preparation of k-casein

K-casein was prepared from acid casein by the urea-sulfuric acid method of Zittle and Custer.<sup>5)</sup>

### III. Immobilization method

Photo-crosslinkable resin prepolymer (ENT-110) was synthesized from polyethylene glycol and methacrylic acid was obtained from Kansai Paint Co.. Immobilized chymosin was prepared by the method described by FUKUI and TANAKA.<sup>6)</sup> The procedure was as follows: One part of photo-crosslinkable resin prepolymer was mixed with 0.02 parts of an initiator, benzoin ethyl ether, and melted by heating at 50°C. To the mixture was added 1.5 parts of the chymosin solution in 0.1 M phosphate buffer (pH 6.0) (enzyme, 0.0015 part). The mixture was laid on a sheet of transparent polyester, covered with the same kind of sheet and illuminated with a Toshiba Chemical Lamp FL20S-BL. Immobilized chymosin thus prepared (thickness, 0.7 mm) was cut into small pieces (5×5 mm).

### IV. Analysis of immobilized chymosin

#### 1. Assay of protein

Protein was measured by the method of LOWRY *et al.*<sup>7)</sup>

#### 2. Assay of chymosin activity

Chymosin and immobilized chymosin products were incubated for 20

min at 30°C with 0.5% k-casein in a 0.1 M sodium citrate buffer of pH 5.3. The reaction was stopped by the addition of trichloroacetic acid to a final concentration of 5% (W/V). After it was filtered with Toyo filter paper 5 B, liberated NPN (nonprotein nitrogen) in the filtrate was measured by the method of LOWRY *et al.*. The chymosin activity of the immobilized chymosin product was expressed in terms of relative activity (percentage of that of native chymosin).

### 3. Assay of stability of immobilized chymosin

Immobilized chymosin product was washed with 0.01 M HCl, 0.01 M acetate buffer of pH 4.1, 0.01 M acetate buffer of pH 5.4, 0.01 M phosphate buffer of pH 6.2 or 0.01 M phosphate buffer of pH 7.0 and then was resuspended in 8 ml of each solution respectively and stored at 4°C for 3 days. After storing, chymosin activities were measured.

### 4. Assay of stability of immobilized chymosin

The immobilized chymosin product was washed with a 0.01 M phosphate buffer of pH 6.2 and then resuspended in 8 ml of the same buffer. After storing for a definite period (0, 10, 30, 60, 120) at 4°C, samples were filtered and chymosin activities were measured.

## Results and Discussion

### 1. Effects of immobilization conditions

To optimize the immobilization conditions for chymosin, several factors were examined.

Fig. 1 shows the effect of illumination time. The lower activity was obtained by a relatively long period of illumination. Inactivation of native chymosin did not occur in a 15 min illumination treatment, and tightly crosslinked gels can be obtained in proportion to the illumination time. Therefore the lower activity obtained may be due to the tightly cross-linked gels, which could lower the diffusion rate of the substrate.

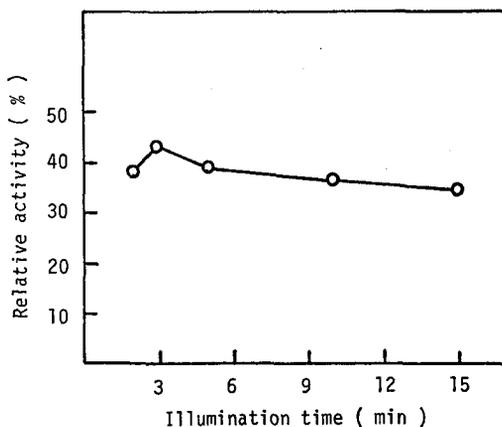


Fig. 1. Effect of illumination time on relative activity of immobilized chymosin.

The activities of corresponding amounts of native chymosin were expressed as 100%.

Based on enzyme activity and mechanical property, an illumination time of 3 min was selected.

The effects of enzyme concentration on relative chymosin activity are shown in Fig. 2. The relative chymosin activity was in inverse proportion to enzyme concentration, although the absolute activity increased. Thus, 5 mg/g resin of the chymosin was employed. Leakage of chymosin from the gels was not observed under the conditions employed.

## 2. Properties of immobilized chymosin

The activities of native chymosin and immobilized chymosin were measured at various temperatures in the range of 30–60°C. The results are shown in Fig. 3. The figures indicate that the optimum temperature for immobilized chymosin was higher than that for the native

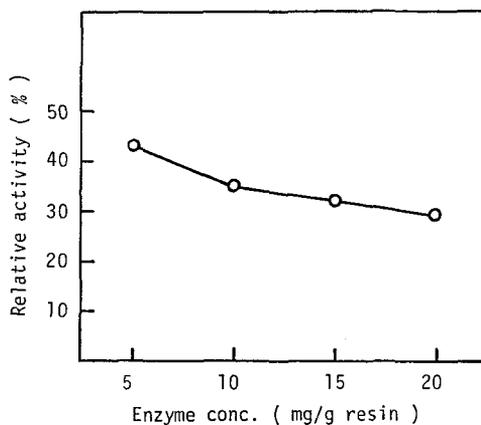


Fig. 2. Effect of enzyme concentration on relative activity of immobilized chymosin.

The activities of corresponding amounts of native chymosin were expressed as 100%.

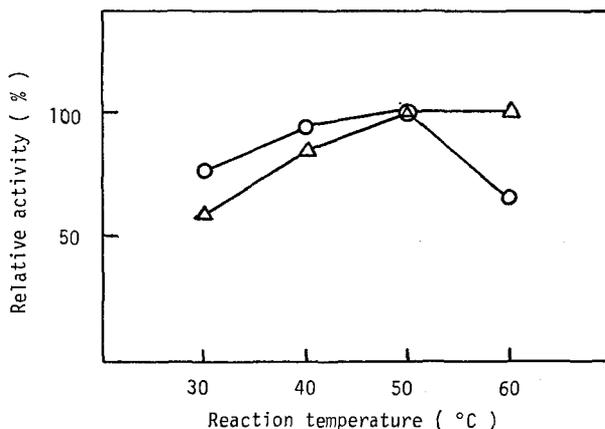


Fig. 3. Effect of temperature on the activity of native chymosin and immobilized chymosin.

The enzyme reaction was carried out under standard conditions modified by varying the temperature. The activities obtained at 50°C were taken as 100%. (○), native and (△), immobilized chymosin.

chymosin. The optimum temperature was 50°C for the native chymosin, but a higher temperature seemed more favorable for the immobilized chymosin. Sometimes the enzyme shows a tendency to change its optimum reaction temperature because of immobilization. If the optimum temperature shifts to a much higher temperature because of immobilization, it would be convenient for industrial application.

To investigate the effect of pH on storage stability, native chymosin and immobilized chymosin were stored for 3 days at pH 2.0–7.0 and 4°C, and chymosin activity was measured. The results are shown in Fig. 4. The present result shows that native chymosin was unstable at pH 4.1, but FOLTMANN<sup>8,9</sup> reported that chymosin was unstable around pH 3.5, and at pH 3.5 A-rennin undergoes a limited proteolysis resulting in the formation of a component which in chromatograms is eluted together with the C-fraction. In our experiments, similar phenomena were observed. On the other hand, immobilized chymosin was remarkable stable at pH below 5.4.

The heat stability of the immobilized chymosin was determined. Both native chymosin and immobilized chymosin were incubated at pH 6.0 at

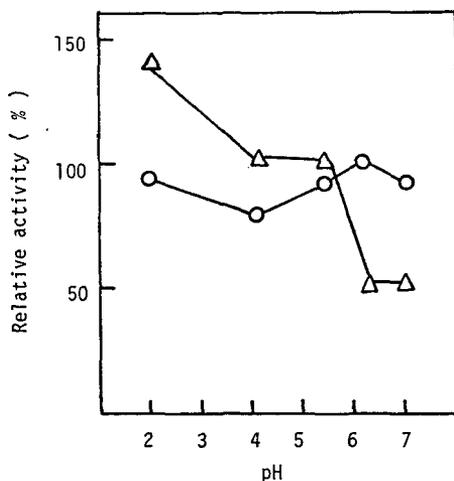


Fig. 4. Effect of pH on the storage stability of native chymosin and immobilized chymosin.

After storage for 3 days at 4°C, the enzyme activities were measured under standard conditions. (○), native and (△), immobilized chymosin.

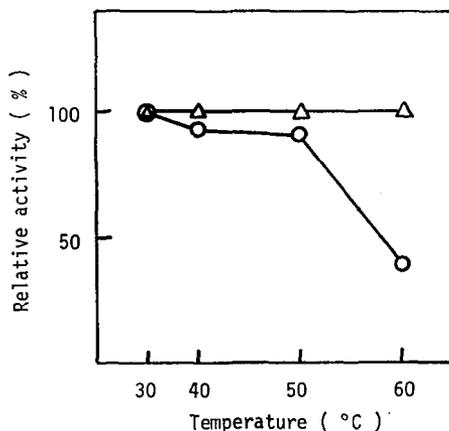


Fig. 5. Effect of heat treatment on relative activity of native chymosin and immobilized chymosin.

After incubation was performed at indicated temperature for 15 min, the enzyme activities were measured under standard conditions. The activities obtained at 30°C were taken as 100%. (○), native and (△), immobilized chymosin.

various temperature for 15 min, and then the enzyme activities were measured at 30°C with a 0.5% k-casein solution as substrate. The results are shown in Fig. 5. It can be seen that, at least during the 15 min studies, the immobilized chymosin was more stable against higher temperatures than the native chymosin. That is, immobilized chymosin retained 100% of its initial activity in a range of 30–60°C, but native only retained 38% at 60°C. Thus, immobilization improved heat stability. OHMIYA *et al.*<sup>10</sup> investigated rennet immobilized by anion exchange resin, and revealed that heat stability was obviously increased by immobilization.

Time-course of heat stability of native chymosin was also studied. As native chymosin was relatively stable at temperatures below 50°C (Fig. 5),

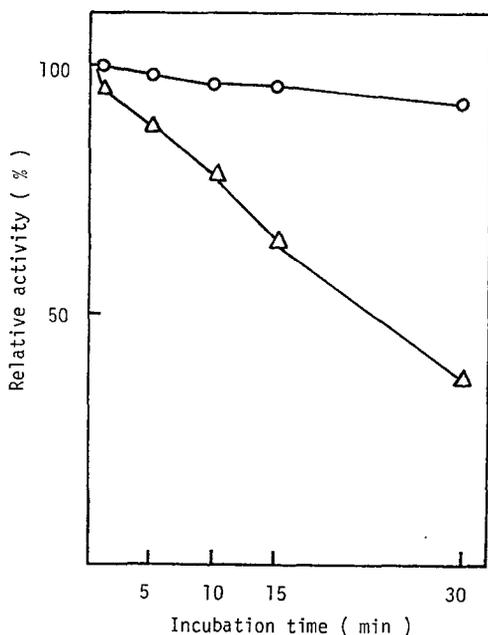


Fig. 6. Heat-stability curve of native chymosin.

Chymosin was dissolved in 0.1M acetate buffer of pH 5.3 (O) and 0.1M phosphate buffer of pH 6.0 (Δ), at a concentration of 0.05% and the solution was incubated at 55°C for indicated times. After incubation was performed, the enzyme activities were measured under standard conditions.

a temperature of 55°C was selected. From the results shown in Fig. 6, it was observed that the rate of inactivation at pH 6.0 was considerably higher than at pH 5.3.

The stability of the immobilized enzyme is the most important factor when immobilized enzymes are used in the industrial field. Table 1 shows the time-course of the leakage of chymosin at pH 6.2 and 4°C. As shown

TABLE 1. Time-course of leakage of chymosin from photo-crosslinkable resin product at pH 6.2

Time (min)	Chymosin activity (%)*	
	Solid material	Filtrate
0	100	0
10	100	0
30	100	0
60	100	0
120	120	0

\* % of activity of the solid material of the sample at zero time.

in Table 1, there was no leakage of chymosin from the gels under the conditions in this experiments, and enzyme activity remained 100% of its initial activity. In our previous paper<sup>11</sup>, we prepared the immobilized chymosin by Sepharose (2 B, 4 B, 6 B) and aminoethylcellulose, and revealed that both products significantly released chymosin into solution. Immobilized chymosin products prepared in the present experiment were a remarkable improvement in comparison with these products.

The stability of immobilized chymosin with repeated enzymatic reaction was examined. Fig. 7 shows the characteristics of decay. The rate of loss was logarithmic with the time of operation. Probably it may be due to the accumulation of a casein layer on the catalyst. This layer may prohibit the enzyme-substrate interaction. The relatively rapid loss of enzyme activity of immobilized proteases has been shown in earlier reports. That is, FERRIER *et al.*<sup>12</sup> reported a rapid accumulation of nitrogenous materials on pepsin-glass during the treatment of skim milk with immobilized pepsin. CHERYAN *et al.*<sup>13</sup> made similar observations and suggested that accumulation of  $\kappa$ -casein or glycopeptides on the glass-enzyme particle caused the rapid decay of activity. TANAKA *et al.*<sup>14,15</sup> investigated catalase and invertase immobilized by photo-crosslinkable resin prepolymers, and demonstrated that im-

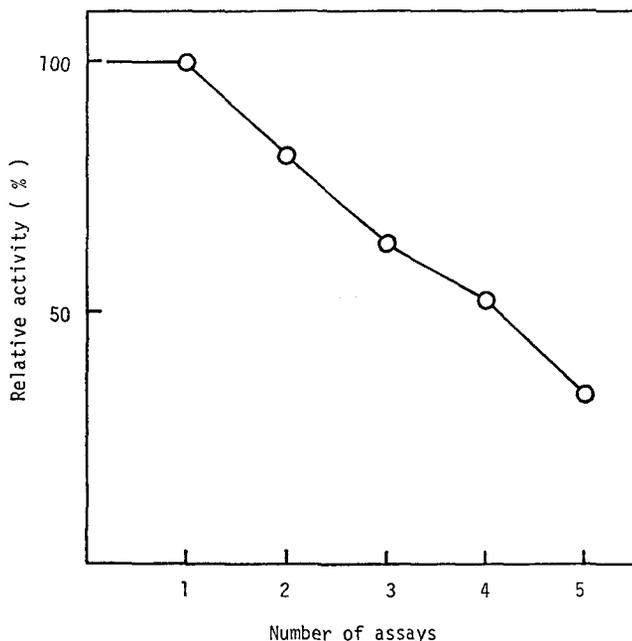


Fig. 7. Decay of immobilized chymosin upon exposure to  $\kappa$ -casein.

mobilized invertase was stable over 30 batch reactions (5 hr operational period) without loss of activity, but in the case of immobilized catalase, the decrease of enzyme activity was marked.

Regeneration would allow reuse of a spent catalyst, a significant economic improvement. But regeneration of spent catalysts was only partially successful. Further research should be conducted into regeneration of catalyst activity. Experiments in these areas are in progress.

Recently proteolytic enzymes have been immobilized by various methods, and enzymatic coagulation of milk and its application to cheese manufacture were studied by many researchers. Green and Crutchfield<sup>10</sup> investigated immobilized chymosin and its application for continuous curd production, but their results proved unsuitable for the manufacture.

Based on the results in this paper, chymosin immobilized with photo-crosslinkable resin prepolymers will be useful in continuous curd production.

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