STUDIES ON THE IMMOBILIZED CHYMOSIN PART II

Preparation and Properties of Immobilized Chymosin by DEAE-Sephadex A-25, Amberlite IR-45, Controlled Pore Glass (CPG-10) and Polyacrylamide Gel

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Synopsis

Immobilized chymosin was prepared by binding chymosin to DEAE-Sephadex A-25, Amberlite IR-45 and Controlled Pore Glass (CPG-10). Also, chymosin was immobilized in crosslinked polyacrylamide gel. Some properties of the preparations —yield, activity, stability with pH and time-course of leakage of chymosin— were investigated.

In coupling 5 mg of chymosin to 100 mg of DEAE-Sephadex A-25, Amberlite IR-45 and CPG-10, their yields were 66.6 mg, 72.0 mg, and 85.5 mg respectively; their preparations contained 32.0%, 38.2% and 33.0% of enzyme protein; and their specific activities were 81.2%, 80.4% and 20.0% of the native chymosin. The activity of chymosin immobilized by polyacrylamide gel was very low.

DEAE-Sephadex A-25 product and Amberlite IR-45 product were similarly stable over the range pH 4.1–7.0 and showed little leakage of chymosin from the products, but the CPG-10 product showed a significant loss of activity and a slow leakage of chymosin into solution at certain pH values during storage.

Among support materials tested, Amberlite IR-45 and DEAE-Sephadex A-25 were best as carriers for chymosin.

Immobilization of enzymes has many advantages: easy removal after enzyme reaction, continuous enzymatic process, increase in enzyme stability, and economical use of the enzymes. Moreover it may be possible to reveal the mode of action of enzymes by using immobilized enzymes. From these points of view, immobilization of enzymes has been extensively studied, and

has been reviewed by several authors[1~3].

The application of immobilized enzymes to milk and milk products has been studied in the hydrolysis of lactose (lactase), the coagulation of milk in cheese making (chymosin, pepsin), the removal of residual hydrogen peroxide used in pasteurization of milk (catalase) and so on. But the application of immobilized proteases to cheese making is only at an early stage, and further studies are necessary in the future.

In a previous paper[4] the authors prepared the immobilized enzymes binding chymosin to Sepharose (2 B, 4 B, 6 B) and aminoethylcellulose, and revealed that both products significantly released chymosin into solution during storage. In this paper, we deal with DEAE-Sephadex A-25, Amberlite IR-45, Controlled Pore Glass (CPG-10) and Polyacrylamide Gel.

Materials and Methods

1. 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.*[5]

2. Preparation of K-casein

K-casein was prepared from acid casein by the urea-sulfuric acid method of Zittle and Custer.[6]

3. Preparation of immobilized chymosin

Immobilized chymosin was prepared by binding chymosin to DEAE-Sephadex A-25, Amberlite IR-45 and Controlled Pore Glass (CPG-10). Moreover, chymosin was immobilized in crosslinked polyacrylamide gel.

1) Preparation of DEAE-Sephdex A-25 product

DEAE-Sephadex A-25 (bead type, 40 to 120 μ. 3.5$\pm$0.5 meq/g) was obtained from Pharmacia (Uppsala, Sweden). After DEAE-Sephadex A-25 was washed with distilled water, 0.5 N NaOH and distilled water, it was equilibrated with 0.1 M phosphate buffer of pH 6.0, and chymosin was coupled to the activated resin as follows. At 4°C, DEAE-Sephadex A-25 was stirred with chymosin solution in phosphate buffer for 3 hr and washed with the same buffer until no further chymosin was detected.

2) Prepartion of Controlled Pore Glass (CPG-10) product

CPG-10 (80 to 120 mesh) was obtained from Electro-Nucleonics, Inc. (Fairfield, N. J.). Chymosin was coupled by the method described by P. J. ROBINSON *et al.*[7]. The glass was immersed in a 2% solution of 3-amino-

propyltriethoxysilane in acetone and allowed to stand at 45°C for 24 hr. Aminoalkylsilane glass was stirred in a cold 1% aqueous solution of glutaraldehyde for 30 min. The derivative was washed with distilled water and was stirred with chymosin solution in 0.1 M phosphate buffer, pH 6.0 for 2 hr at 4°C. After the product was kept standing overnight, it was washed with phosphate buffer until no further chymosin was detected.

3) Preparation of Amberlite IR-45 (OH) product

Amberlite IR-45 (14-52 mesh) was obtained from Orugano Inc. (Tokyo, Japan). The product was prepared by the method described by K. Y. PARK and C. D. LIMA.[8] Amberlite IR-45 was washed by stirring acetone, and then with distilled water, 1 N HCl, distilled water, and finally with 1 N NaOH. After washing with distilled water, it was equilibrated with 0.1 M phosphate buffer, pH 6.0. The activated resin was then mixed with chymosin solution. After stirring the resin and chymosin mixture for 2 hr at 4°C allowing it to stand overnight, it was washed with phosphate buffer until no further chymosin was detected.

4) Preparation of polyacrylamide gel product[9]

1000 mg of acrylamide monomer and 55 mg BIS (N, N'-methylene-bis-acrylamide) were added to 10 ml of a solution of 50 mg chymosin dissolved in 0.1 M phosphate buffer of pH 6.0, and then, 0.6 ml of 5% DMAPN (β-dimethylaminopropionitrile) and 5% potassium persulfate solution were added to the mixture was left at room temperature for 60 min. The gel particles were throughly washed with phosphate buffer until no further chymosin was detected.

4. Analysis of immobilized chymosin

1) Determination of dry weights of immobilized chymosin prodncts

Dry weights were determined as follows. A known volume of immobilized chymosin in suspension was washed with distilled water, ethanol and ether on a glass filter and then dried with an electric drier (99±1°C).

2) Determination of amount of chymosin bound

The amount of chymosin bound was determined from the nitrogen content of the products. Nitrogen analyses were carried out by the micro-method of C. A. LANG.[10]

3) Determination of chymosin activity of immobilized chymosin products

Chymosin and immobilized chymosin products were incubated for 20 min at 30°C with 0.5% K-casein in 0.1 M sodium citrate buffer of pH

5.3, and 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 (non-protein nitrogen) in the filtrate was measured by the method of LOWRY *et al.*[11]. The chymosin activity of the immobilized chymosin products was expressed in terms of relative activity (percentage of that of native chymosin).

4) Determination of stability of immobilized chymosin products

Each 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 and 0.01 M phosphate buffer of pH 7.0, and then resuspended in 8 ml of each solution and stored at 4°C for 3 days. After storage, each suspension was filtered. Chymosin activities in the filtrate and the solid material were measured.

5) Determination of time-course of leakage of chymosin from immobilized chymosin products at pH 6.2

Each immobilized chymosin product was washed with 0.1 M phosphate buffer of pH 6.2, and then resuspended in 8 ml of the same buffer. After storage for a definite period (0, 10, 30, 60, 120 min) at 4°C, samples were filtered and chymosin activities in the filtrate and the solid material were measured.

Results and Discussion

Extent of coupling of chymosin to carriers and chymosin activities of immobilized chymosin products

Chymosin was coupled with three kinds of insoluble carriers. 50 mg of purified chymosin was added to 1 g of an insoluble carrier. The amount of products, the extent of the coupling of chymosin and the activities of immobilized chymosin products are shown in Table 1.

TABLE 1. Properties of immobilized products of Chymosin

Support	Yield of products (mg)	Chymosin content (%)	Activity of native chymosin (%)
DEAE-Sephadex A-25	66.6	32.0	81.2
Amberlite IR-45	72.0	38.2	80.4
CPG-10	85.5	33.0	20.0

* At 4°C, 5 mg of chymosin was coupled to 100 mg of support materials.

The results indicated that the coupling rates of chymosin with Amberlite IR-45, CPG-10 and DEAE-Sephadex A-25 were 38.2%, 33.0% and 32.0% respectively.

The amount of enzyme bound to an insoluble carrier generally depends on the kind of enzyme and support material, the method of immobilization and so on. SUKEGAWA and TAKAHASHI[12] investigated lactase immobilized by DEAE-Sephadex A-25 and porous glass beads, and revealed that the coupling rate of lactase reached a maximum when about 20 mg of lactase was added to 1 g of the insoluble carriers.

As shown in Table 1, the relative activity of the CPG-10 product was only 20.0%, but the activities of the Amberlite IR-45 product and the DEAE-Sephadex A-25 product were 80.4% and 81.2% respectively.

WOYCHICK *et al.*[13] reported that the activity of immobilized lactase fixed by porous glass beads was 75%. ROYER *et al.*[14] also reported the enzyme activity was 57-67% in the case of pronase. The present result in the case of CPG-10 product showed a relatively low activity in comparison with their results. TOSA *et al.*[15] reported that the activity of aminoacylase immobilized by DEAE-Sephadex A-25 was 59%, and Y. K. PARK[16] reported that the activity of immobilized glucoamylase coupled with Amberlite IR-45 was 12-20%. The present results in both cases showed higher activities than their results.

The activity of chymosin immobilized by polyacrylamide gel was very low, though it contained 57.0% chymosin.

The immobilization of enzymes has progressed rapidly in recent years, and immobilized enzymes can be prepared by a wide variety of methods. But it should be recognized that no single immobilization technique is ideal for all enzymes.

In comparison with the native enzyme, enzyme activity and substrate specificity are changed by immobilization; its phenomenon occurs most remarkably on the high molecular weight substrates.

Immobilization of enzymes by an ionic bond is the most widely studied method. This method offers the advantage of simplicity of preparation procedure under very mild conditions. The enzyme is not modified chemically, and hence the immobilized enzymes show a tendency to have a relatively high activity. Another feature of enzyme absorption is the reversibility factor. Leakage of enzyme from the support material can occur if there is a change in such enviromental factors as pH and temperature. Therefore, the ionic strength and pH of the reaction solution must be carefully controlled. While this feature could be a problem, it has been used to advantage

in the regeneration of immobilized enzymes.

Covalent bonding of enzymes with support materials is generally the most frequently used method of enzyme immobilization. In general, enzymes immobilized by this method show a tendency to have relatively low activity. Destruction of the three-dimentional conformation of enzymes and steric hindrance effects may be significant. A major advantage of this method is that there is little or no leakage of enzyme from the support material.

In the present experiment, the chymosin activity of the CPG-10 product was lower than those of the DEAE-Sephadex A-25 product and the Amberlite IR-45 product.

Stability of immobilized chymosin with pH and time-course of leakage of soluble chymosin from immobilized chymosin products

The stability of the immobilized enzyme is the most important factor when immobilized enzymes are used in the industrial field. The effects of pH and storage period on immobilized enzyme activity were investigated.

1) Stability of immobilized chymosin products with pH

To investigate the stability, each product was stored for 3 days at pH 4.1-7.0 and 4°C, and chymosin activities in the filtrate and the solid material were assayed. The results are shown in Table 2 to Table 4.

As shown in Table 2, the DEAE-Sephadex A-25 product was stable between pH 4.1 and pH 7.0, and 90% of the chymosin activity remained in insoluble form.

TABLE 2. Stability of DEAE-Sephadex A-25-chymosin with pH

Storage solution	Chymosin activity*		% of activity remaining in insoluble form
	Solid material	Filtrate	
0.01 M-HCl	81	71	53
0.01 M-Acetate buffer, pH 4.1	102	11	90
0.01 M-Acetate buffer, pH 5.4	100	11	90
0.01 M-Phosphate buffer, pH 6.2	98	11	90
0.01 M-Phosphate buffer, pH 7.0	98	11	90

* % of activity of the solid material of the sample stored at pH 5.4.

The result for Amberlite IR-45 is shown in Table 3. The Amberlite IR-45 product was stable between pH 5.4 and pH 7.0, and was the most stable among the three products tested.

TABLE 3. Stability of Amberlite IR-45-chymosin with pH

Storage solution	Chymosin activity*		% of activity remaining in insoluble form
	Solid material	Filtrate	
0.01 M-HCl	64	19	77
0.01 M-Acetate buffer pH 4.1	93	14	87
0.01 M-Acetate buffer, pH 5.4	100	11	90
0.01 M-Phosphate buffer, pH 6.2	102	12	89
0.01 M-Phosphate buffer, pH 7.0	12	12	89

* % of activity of the solid material of the sample stored at pH 5.4.

As shown in Table 4, the CPG-10 product was stable only at pH 5.4 in contrast with DEAE-Sephadex A-25 and Amberlite IR-45. Moreover, it showed a remarkable leakage of chymosin at pH 2.0 and pH 4.1 and showed a significant loss of activity at pH 6.2 and pH 7.0.

TABLE 4. Stability of CPG-10-chymosin with pH

Storage solution	Chymosin activity*		% of activity remaining in insoluble form
	Solid material	Filtrate	
0.01 M-HCl	48	62	44
0.01 M-Acetate buffer, pH 4.1	76	55	58
0.01 M-Acetate buffer, pH 5.4	100	36	74
0.01 M-Phosphate buffer, pH 6.2	67	19	78
0.01 M-Phosphate buffer, pH 7.0	67	14	83

* % of activity of the solid material of the sample stored at pH 5.4.

Each product showed a maximum stability and a minimum leakage of chymosin at pH 5.4. On the whole, the stability of immobilized chymosin products prepared in the present experiment was improved remarkable in comparison with the Sepharose and Aminoethylcellulose products studied in our previous paper.

2) Stability of immobilized chymosin products with time-course of leakage of chymosin

Tables 5 to 7 show the time-course of the leakage of chymosin from each product at pH 6.2 and 4°C.

As shown in Tables 5 and 6, there was little leakage of chymosin from the products prepared by DEAE-Sephadex A-25 and Amberlite IR-

45 with time-course. But the latter showed a relatively higher initial leakage of chymosin (when time is zero) than the former.

On the other hand, CPG-10 product showed a significant leakage of chymosin with time-course, as shown in Table 7. LINE *et al.*[17] reported

TABLE 5. Time-course of leakage of chymosin from DEAE-Sephadex A-25 product at pH 6.2

Time (min)	Chymosin activity (%)*	
	Solid material	Filtrate
0	100	10
10	100	10
30	100	11
60	100	13
120	100	13

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

TABLE 6. Time-course of leakage of chymosin from Amberlite IR-45 product at pH 6.2

Time (min)	Chymosin activity (%)*	
	Solid materil	Filtrate
0	100	35
10	100	35
30	93	37
60	93	37
120	93	37

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

TABLE 7. Time-course of leakage of chymosin from CPG-10 product at pH 6.2

Time (min)	Chymosin activity (%)*	
	Solid material	Filtrate
0	100	57
10	94	61
30	83	64
60	80	67
120	78	68

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

that when the enzyme reaction was carried out for continuously for 30 days at 6°C using a column packed with immobilized pepsin fixed to porous glass beads, enzyme activity remained at 100% throughout the experiment.

Recently the problem of a world shortage of rennet in the field of dairy products has given rise to a number of papers on the immobilization of proteolytic enzymes, enzymic coagulation of milk by immobilized proteolytic enzymes and their application to cheese manufacturing. GREEN and GRUTCHFIELD[18] investigated immobilized chymosin by fixation to Sepharose and Aminoethylcellulose, and continuous curd production. However, both products significantly released chymosin into solution during storage, so they are not yet suitable for industrial use. Further research should be conducted to produce an immobilized chymosin with higher stability than native chymosin. Experiments in these areas are in progress.

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