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Optimization of Protease Immobilization by Covalent Binding Using Glutaraldehyde

HEE JEONG CHAE,[1,*] MAN-JIN IN,[1] AND EUI YONG KIM[2]

[1]R&D Center, Daesang Corp., 125-8 Pyokyo-ri, Majang-myun, Icheon, Kyoungki 467-810, Korea; and [2]Department of Chemical Engineering, The University of Seoul, Seoul 130-743, Korea

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ABSTRACT

Immobilization of a protease, Flavourzyme, by covalent binding on various carriers was investigated. Lewatit R258-K, activated with glutaraldehyde, was selected among the tested carriers, because of the highest immobilized enzyme activity. The optimization of activation and immobilization conditions was performed to obtain high recovery yield. The activity recovery decreased with increasing carrier loading over an optimal value, indicating the inactivation of enzymes by their reaction with uncoupled aldehyde groups of carriers. The buffer concentrations for carrier activation and enzyme immobilization were optimally selected as 500 and 50 m*M*, respectively. With increasing enzyme loading, the immobilized enzyme activity increased, but activity recovery decreased. Immobilization with a highly concentrated enzyme solution was advantageous for both the immobilized enzyme activity and activity recovery. Consequently, the optimum enzyme and carrier loadings for the immobilization of Flavourzyme were determined as 1.8 mg enzyme/mL and 0.6 g resin/mL, respectively.

Index Entries: Protease; immobilization; glutaraldehyde; covalent binding; carrier loading; enzyme loading.

INTRODUCTION

Protein hydrolysates are widely utilized in food production, particularly in soups, sauces, and processed food *(1–2)*. The industrial production

* Author to whom all correspondence and reprint requests should be addressed.

of protein hydrolysate is carried out by the hydrolysis of protein by acids or alkalis. A biological method using proteases has several advantages, including mild reaction conditions and avoidance of undesirable byproduct. Other uses of proteases in the food industry includes the development of new functional properties of proteins *(3)*. However, the use of the enzymatic method has been limited, because of relatively high enzyme cost. Most industrial enzymatic processes are carried out batchwise, using soluble enzymes. In order to reuse enzymes after enzyme reaction, and to enhance their stability, the immobilization of enzymes, using an insoluble carrier, has been widely studied *(4)*. The use of immobilized enzymes is advantageous because of low-cost effect of reusing the enzymes, easy product separation, the possibility of continuous or semicontinuous operation, and enhanced enzyme stability, in some cases *(5)*.

General immobilization methods include ionic binding, covalent binding, adsorption, and membrane entrapment *(5)*. Immobilization of proteases by covalent binding is particularly attractive, because there is less loss of enzyme *(6–7)*. Some bifunctional agents, such as glutaraldehyde, cyanogen bromide, and carbodiimide, are necessary to induce the covalent binding between enzyme molecules and carriers.

In the present study, various matrices were tested for the immobilization of an exopeptidase, Flavourzyme® (Novo Nordisk, Bagsvaerd, Denmark). The effects of immobilization conditions, such as carrier activation, buffer concentration, and carrier and enzyme loadings, were investigated, to maximize the activity of immobilized enzyme and activity recovery.

MATERIALS AND METHOD

Materials

The protease Flavourzyme, originated from *Aspergillus oryzae*, was obtained from Novo. Silica gel and glass beads were purchased from Merck (Darmstadt, Mannheim, Germany) and WAB (Basel, Switzerland), respectively. Diaion™ HP20 and SP206 were obtained from Mitsubishi Chemical (Tokyo, Japan). Lewatit® R258-K (Bayer AG, Leverkusen, Germany) was used as immobilization carrier after activation. Glutaraldehyde (GA), cyanogen bromide (CNBr), polyethyleneimine (PEI), and 1,4-benzoquinone (BQ) were purchased from Sigma (St. Louis, MO).

Activation of Carriers

Various carriers were treated with appropriate reagents for the activation, as described in the literature: Diaion HP20 with GA *(8)* or PEI-GA *(9)*;

Diaion SP206 with PEI-GA *(9)*; glass bead and silica gel 60 with PEI-GA *(9)* or CNBr *(4)*; Lewatit R258-K with GA or BQ *(10)*. For example, activation of Lewatit R258-K using glutaraldehyde was carried out as follows: 10 g of Lewatit R258-K were washed twice with 20 mL phosphate buffer (pH 7.0). The resins were activated with 100 mL glutaraldehyde solution at 4°C. The suspension of the resins in the glutaraldehyde solution was stirred at 150 rpm during the activation. The contact time, glutaraldehyde, and buffer concentrations were determined by the experiments. The resins were washed with the same buffer, and then stored at 4°C until immobilization.

Enzyme Immobilization

The enzyme powder was dissolved in phosphate buffer (pH 7.0) to make a concentration of 0.6 mg/mL. The activated carriers were suspended in the enzyme solution. The mixture was stirred for 4 h at 4°C, to allow the coupling reaction between the enzyme and the carriers. After this reaction, the unbound enzyme fraction was collected, and the residual activity was determined.

Enzyme Activity Assay

Activities of both soluble and immobilized enzymes were measured by leucine aminopeptidase (LAP) assay method, by means of a kit from Sigma. Soluble enzyme activity was estimated by the addition of 0.5 mL of the protease solution to the assay mixture containing L-leucyl-β-naphthylamide, previously incubated at 37°C. The assay mixture was stirred for 1 h at the same temperature. One LAP unit was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of L-leucyl-β-naphthylamide to liberate L-leucine/min.

The activity of immobilized enzyme was estimated by the addition of protease-loaded carriers to the assay mixture. The immobilized carriers of 10 mg were placed in a 100-mL baffled flask containing the assay mixture (5 mL). The activity of immobilized enzyme (U_{imm}) was divided by the wet weight of carrier to calculate the activity of immobilized enzyme: U_{imm}/R (U/g resin).

Protein Assay

The protein concentration was determined by Bradford method *(11)*, with bovine serum albumin as a protein standard. Bound protein was determined by the difference between the initial and residual protein concentrations.

RESULTS AND DISCUSSION

Screening and Activation of Carrier

Various types of immobilization carrier were tested for the immobilization of Flavourzyme (Table 1). The activity of immobilized enzyme was measured by assaying the enzyme-loaded carriers. When Lewatit R258-K was used after being treated with bifunctional agents, both the immobilized enzyme activity (U_{imm}/R) and immobilized protein (P_{imm}/R) were the highest among the tested carriers. The specific activity (SA), which was defined as immobilized enzyme activity per weight of immobilized protein (U_{imm}/P_{imm}), was also the highest in Lewatit R258-K. This resin has been selected as a carrier, because it has additional advantages of high porosity and mechanical strength. Lewatit R258-K is a macroporous polymer in spherical bead form, whose functional group is benzylamine. Lewatit R258-K had a surface area of 50 m^2/g dry wt, and an average diameter of 0.5 mm. The porosity of the Lewatit R258-K plays an important role in the protease immobilization. It was essential to activate the carrier with glutaraldehyde or 1,4-benzoquinone, prior to immobilization. When the carrier was activated by bifunctional agents, glutaraldehyde showed a lower immobilized protein (P_{imm}/R) than 1,4-benzoquinone. However, glutaraldehyde provided a slightly higher immobilized enzyme activity than 1,4-benzoquinone. The enzyme activity seemed to be kept more stable in glutaraldehyde-activated resin. Lewatit R258-K treated with glutaraldehyde was thus selected in further experiments. The amine groups of the enzymes (NH_2-Enz) and carriers (NH_2-R) were covalently bound through glutaraldehyde.

Different variables for the activation of Lewatit R258-K by glutaraldehyde were examined to maximize immobilization yield. First, the influence of glutaraldehyde concentration was investigated, but none was observed when the concentration was above 1% (Fig. 1). The contact time over 2 h had no effect on the activation of Lewatit R258-K (data not shown). The carriers were reacted with 2.5% glutaraldehyde in phosphate buffer (pH 7.0), with the ratio of 1 g carrier per 10 mL of glutaraldehyde solution in further experiments.

Effective Factors on Enzyme Immobilization

Activity recovery (AR) and effectiveness factor (EF) were defined for yield parameters of immobilization as follows:

$$AR = \text{immobilized enzyme activity}/\text{ initially added activity} \times 100(\%)$$

and

$$EF = \text{immobilized enzyme activity}/(\text{initially added activity} - \text{raffinate activity}) \times 100(\%)$$

Table 1
Immobilization of Flavourzyme on Various Carriers by Covalent Binding[a]

Carrier	Activator[b]	Immobilized enzyme activity U_{imm}/R (U/g resin)	Immobilized protein P_{imm}/R (mg/g resin)	Specific activity U_{imm}/P_{imm} (U/mg protein)
Diaion HP20	GA	0.13	1.73	0.08
	PEI-GA	0.08	1.04	0.08
Diaion SP206	PEI-GA	0.21	1.06	0.20
Glass bead	PEI-GA	0.08	1.21	0.07
	CNBr	0.11	0.45	0.24
Silica gel 60	CNBr	0.03	1.13	0.03
	PEI-GA	13.8	0.50	27.6
Lewatit R258-K	GA	75.4	2.20	34.3
	BQ	46.7	2.67	17.5

[a] Added activity, 2725 unit; carrier weight, 5 g; enzyme solution volume, 100 mL.
[b] GA, glutaraldehyde; PEI, polyethyleneimine; CNBr, cyanogen bromide; BQ, 1,4-benzoquinone.

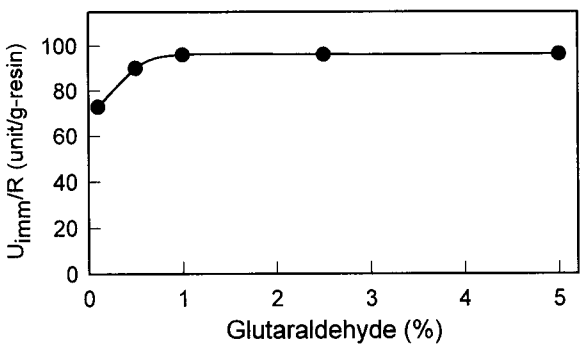

Fig. 1. Effect of glutaraldehyde concentration on the activity of immobilized enzymes. Immobilization conditions: carrier weight, 2 g; volume of enzyme solution, 20 mL; enzyme concentration, 0.6 mg/mL in 50 m*M* phosphate buffer.

Figure 2 shows the pH changes during the activation of carriers and immobilization of enzymes, even though phosphate buffer was used. A sudden change of pH cannot provide sufficient activation of the carriers and has deleterious effects on the enzymes. High activity recovery of immobilization was obtained when the carriers were activated in a highly concentrated buffer (Fig. 3A). The optimal immobilization was obtained at a buffer concentration of 50 m*M* (Fig. 3B). At this concentration of buffer, the specific activity was the highest among the tested concentrations, indicating the favorable state of enzyme immobilization onto the carrier.

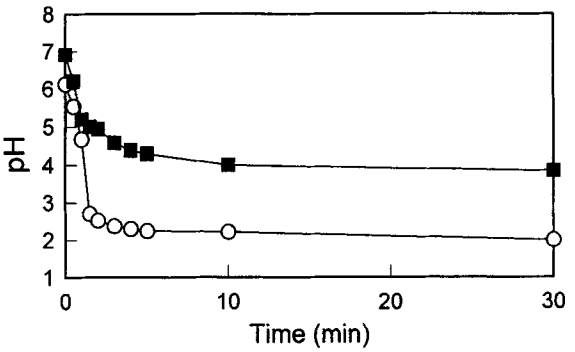

Fig. 2. pH change during the carrier activation (○) and enzyme immobilization (■). Carrier loading, 2 g resin/20 mL; enzyme concentration, 0.6 mg/mL.

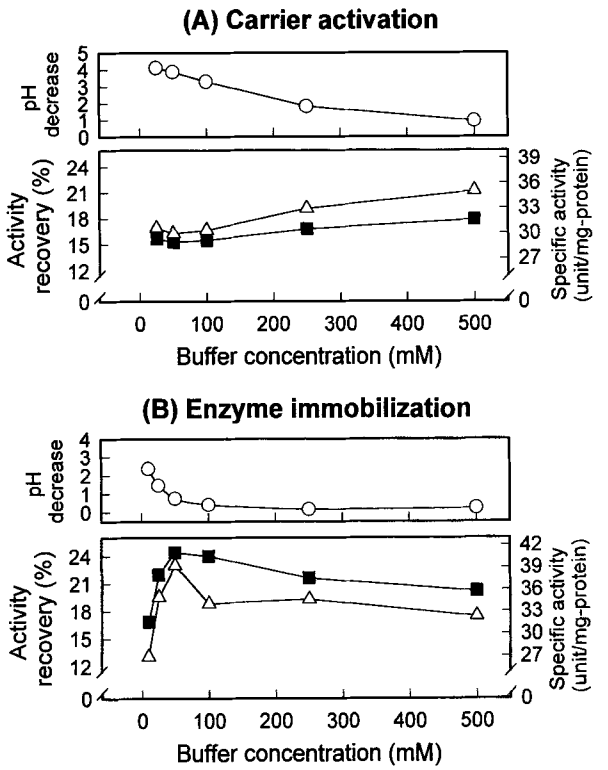

Fig. 3. Effects of buffer concentration on pH change and AR in immobilization during the carrier activation **(A)** and enzyme immobilization **(B).** Carrier loading, 3 g resin/25 mL; enzyme concentration, 0.6 mg/mL. Symbols: (○), pH decrease; (■), AR; (△), SA.

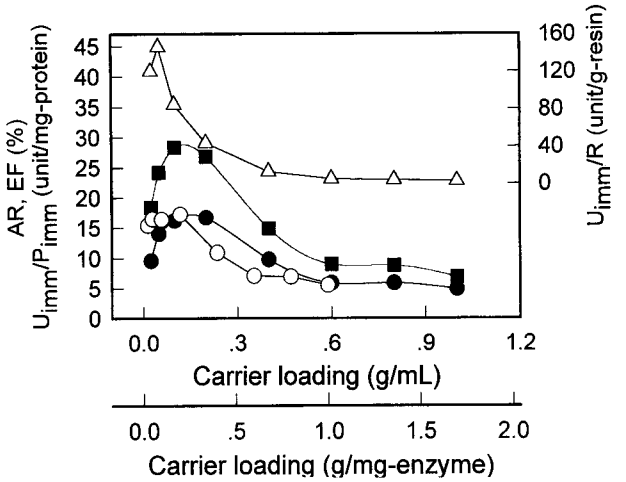

Fig. 4. Effect of carrier loading on the enzyme immobilization. Immobilization conditions: volume of enzyme solution, 20 mL; enzyme concentration, 0.6 mg/mL in 50 m*M* phosphate buffer. Symbols: (●), AR; (○), EF; (■), U_{imm}/P_{imm}; (△), U_{imm}/R.

Further activation and immobilization were carried out using phosphate buffers of 50 and 500 m*M*, respectively.

The effects of carrier loading on the immobilization of Flavourzyme were also investigated (Fig. 4). The activity of immobilized enzyme (U_{imm}/R) decreased with increasing carrier loading, which was probably caused by the competition of carriers toward a given quantity of enzyme molecules. The maximum AR was obtained at a carrier loading of 0.12 g resin/mL (0.2 g resin/mg enzyme with 0.6 mg enzyme/mL). In the range in which carrier loading was less than 0.12 g-resin/mL, the AR was low, despite high U_{imm}/R. In the region of excess carrier (more than the optimal value), the AR and SA (U_{imm}/P_{imm}, U/mg enzyme) decreased with increasing carrier loading. It appeared that the enzymes were inactivated by the neighboring unbound aldehyde group (-CHO) of excess carriers. This was supported by the fact that U_{imm}/P_{imm} decreased, even though the total immobilized protein (P_{imm}) increased according to carrier loading, as shown in Fig. 4. The immobilization occurs by the covalent binding between aldehyde groups of glutaraldehyde and amine groups of the enzyme. Glutaraldehyde plays a role as a spacer arm of the carriers. If the functional groups (-CHO) of the spacer arm are not occupied by the amine groups of enzyme, the unbound groups (excess -CHO) can randomly bind with amine groups of the enzymes. This can be a factor of enzyme inactivation during the immobilization. The previously selected optimum carrier loading might not be the overall optimum, since it was determined at a certain enzyme concentration. Therefore, the effect of enzyme loading should be considered together, along with that of carrier loading.

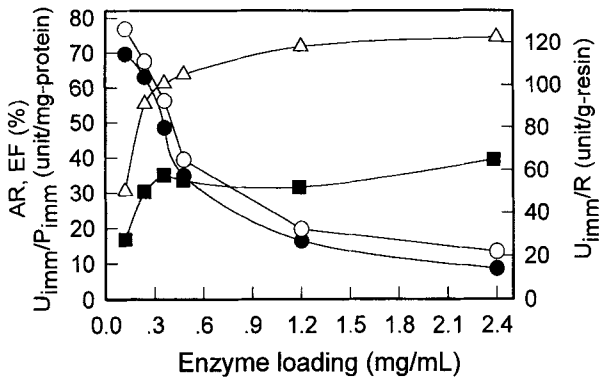

Fig. 5. Effect of enzyme loading on the enzyme immobilization. Immobilization conditions: carrier loading, 3 g resin with 25 mL enzyme solution in 50 m*M* phosphate buffer. Symbols: (●), AR; (○), EF; (■), U_{imm}/P_{imm}; (△), U_{imm}/R.

The preparation of the commercial enzyme, Flavourzyme, may be composed of various materials, such as salt and stabilization agent, to say nothing of protein. The protein content of Flavourzyme used in this experiment was determined as 5.7%. It seemed more reasonable to investigate the effect of protein loading in place of enzyme loading as an effective parameter, because the covalent binding occurs between the carriers and protein molecules in enzyme preparation. However, enzyme loading was selected in this study for convenience: protein loading was assumed to be proportional to enzyme loading. Figure 5 shows the effects of enzyme loading (mg enzyme/mL) on the protease immobilization at the predetermined optimum carrier loading (0.12 g resin/mL). The activity of immobilized enzyme (U_{imm}/R) increased, but AR decreased, with the increase of enzyme loading. In an economic point, the optimum enzyme loading was determined as 0.36 mg enzyme/mL (or 3 mg enzyme/g resin, based on carrier weight), with which an AR of 45% and an immobilized enzyme activity of 105.2 U/g resin were obtained.

The AR and immobilized enzyme activity were measured for various volumes of enzyme solution, given the constant enzyme weight (9 mg) and carrier weight (3 g), as shown in Table 2. Both the immobilized enzyme activity and AR decreased with the increase of enzyme solution volume, which was probably the result of the influence of the degree of contact between the carriers and enzyme molecules. The enzyme solution of high concentration and low volume can give high probability of contact. The immobilized enzyme activity, AR and SA were the highest when the volume of enzyme solution was 5 mL, which is the minimum volume required to soak the carriers. The enzyme concentration (or loading) used in this condition was 1.8 mg/mL, which is the optimum enzyme loading, considering the effect of volume of enzyme solution. Consequently, the

Table 2
Effect of the Volume of Enzyme Solution on the Immobilized Enzyme Activity and Activity Recovery at a Constant Enzyme Weight[a]

Volume of enzyme solution (mL)	Enzyme concentration (mg/mL)	Immobilized enzyme activity U_{imm}/R (U/g resin)	Specific activity U_{imm}/P_{imm} (U/mg protein)	Activity recovery (%)
5.0	1.80	107.1	36.9	43.4
10.0	0.90	99.5	33.7	40.4
15.0	0.60	86.4	28.2	35.2
20.0	0.45	86.7	28.0	35.0
25.0	0.36	80.0	26.3	32.5

[a] Carrier weight, 3 g; enzyme weight, 9 mg.

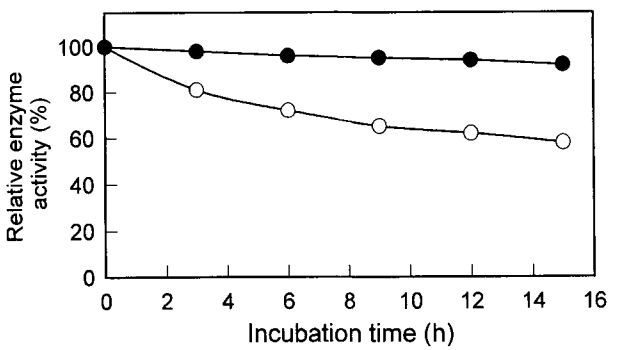

Fig. 6. Thermal stability of immobilized and native proteases at 65°C. The immobilized and native enzyme preparations in phosphate buffer (pH 7.0) were incubated at 65°C. Enzyme samples were withdrawn at various time intervals, and enzyme activities toward L-leucyl-β-naphthylamide were determined. Symbols: (○), native enzyme; (●), immobilized enzyme.

optimum carrier loading was recalculated to 0.6 g resin/mL from the optimum enzyme loading of 3 mg/g resin.

Thermal Stability Test for Immobilized Enzyme

The thermal stability of enzymes is one of the important criteria for long-term and commercial application. The activity of immobilized enzyme is known to be more resistant against heat than that of the native state. The thermal stability of immobilized protease was compared with that of native form by measuring the residual enzyme activity during incubation at 65°C. As shown in Fig. 6, the immobilized proteases could express their activity without a significant loss of activity; the correspond-

ing native proteases lost more than 38% of their activity after 15 h incubation. The higher stability of the immobilized enzyme can be attributed to the prevention of autodigestion and thermal inactivation, which is possibly caused by the fixation of protease on the microsphere surface of the resin *(12)*.

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