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Neutrase Immobilization on Alginate−Glutaraldehyde Beads by Covalent Attachment

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Neutrase, a commercial preparation of *Bacillus subtilis*, was covalently immobilized on alginate−glutaraldehyde beads. Immobilization conditions and characterization of the immobilized enzyme were investigated. Central composite design and response surface methods were employed to evaluate the effects of immobilization parameters, such as glutaraldehyde concentration, enzyme loading, immobilization pH, and immobilization time. Under optimized working conditions (2% alginate, 6.2% glutaraldehyde, 61.84 U mL^{-1} Neutrase, pH 6.2, and 60 min) the immobilization yield was about 50%. The immobilized enzyme exhibited higher K_m compared to the soluble enzyme. The pH−activity profile was widened upon immobilization. The optimum temperature was shifted from 50 to 60 °C, and the apparent activation energy was decreased from 47.7 to 22.0 kJ mol^{-1} by immobilization. The immobilized enzyme also showed significantly enhanced thermal stability.

KEYWORDS: Alginate−glutaraldehyde beads; covalent immobilization; factorial design; Neutrase; response surface methodology

INTRODUCTION

Proteases (EC 3.4.21−24) represent one of the largest groups of industrial enzymes with increasing market demands due to their applications in industrial, biotechnological, medical, and basic research fields (*1*). Nowadays, commercially available crude proteases are used extensively in the food industry to prepare protein hydrolysates with improved nutritional or functional properties (*2, 3*). Manipulation of hydrolysis reaction conditions can be used, to some extent, to define the characteristics of the final hydrolysates. However, the specificities of the enzymes that are used determine the type of peptides produced and, therefore, the properties of specific food protein hydrolysates (*4*).

Neutrase, commercialized by Novozymes, is a bacterial endoprotease produced by fermentation of a selected strain of *Bacillus subtilis*. It attracts considerable interest due to a wide variety of possible applications, for example, in the production of functional food proteins by hydrolysis of mung bean (*5*), corn gluten (*6*), cheese whey protein (*7*), tilapia (fish) (*8*), and soybean (*9*), in the improvement of the texture and sensory properties of dairy products (*10*), and in peptide synthesis in organic media (*11*).

Neutrase immobilization could offer considerable advantages with the possibility of continuous processing, reuse of the enzyme, and reduction of autodigestion. To the best of our knowledge, Neutrase has been already immobilized by physical adsorption on CM-Sephadex C-50 (*10*) and Celite and polyamide (*11*), but there are no studies in the literature reporting the covalent immobilization of Neutrase.

The immobilization procedure on alginate beads is not only inexpensive but also very easy to carry out and provides extremely mild conditions, so that the potential for industrial application is considerable. Although this matrix has been extensively used to immobilize by entrapping, Le-Tien et al. (*12*) have described the covalent immobilization of enzyme on alginate activated with sodium periodate. Yeom and Lee (*13*) have described that hydroxyl groups of alginate can react with the aldehyde groups of glutaraldehyde. Nevertheless, although alginate has been cross-linked with glutaraldehyde to avoid the leakage of material encapsulated in alginate (*14*) and get very compact and stable beads (*15, 16*), the activation of alginate beads with glutaraldehyde prior to enzyme addition has not been reported. Another aspect to be considered is that the immobilization on glutaraldehyde preactived support is quite simple and efficient and in some instances even permits the improvement of enzyme stability by multipoint or multisubunit immobilization (*17, 18*).

The present study demonstrated the covalent immobilization of Neutrase to alginate beads activated with glutaraldehyde. Therefore, the main objectives of this work were to evaluate the effect of the immobilization variables (glutaraldehyde concentration, enzyme loading, immobilization pH, and contact time) to the immobilization process and to obtain the optimum conditions for immobilized Neutrase using response surface methodology. Finally, the properties of the immobilized enzyme

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Table 1. Independent Variables and Their Levels Used for the First Experiment (Central Composite Design 2^4)

		coded variable levels				
independent variable	symbol	$-\alpha$	-1	0	$+1$	$+\alpha$
glutaraldehyde (%, w/v)	X_1	0	2	4	6	8
enzyme loading[a] (U mL^{-1})	X_2	10.28	61.84	113.40	164.96	216.53
immobilization pH	X_3	5.3	6.1	6.9	7.7	8.5
contact time (min)	X_4	10	60	110	160	210

[a] Imobilization condition: 0.6 mL of enzyme solution per gram of support.

such as kinetic behavior, pH and temperature profile, and thermal stability were studied.

MATERIALS AND METHODS

Materials and Reagents. Neutrase 0.5 L was produced by Novozymes A/S (Bagsvaerd, Denmark) and complies with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC). Casein, glutaraldehyde, and Folin−Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO). The immobilization support (sodium alginate) was supplied by Aldrich Chemical Co. (St. Louis, MO). All other chemicals used in the present study were of analytical or better grade.

Alginate−Glutaraldehyde Bead Preparation. The alginate beads used as immobilization support were prepared by dropping 50 mL of 2% (w/v) sodium alginate through a peristaltic pump into 100 mL of a 0.2 M $CaCl_2$ solution under continuous stirring during 12 h and stored at 4 °C in 0.03 M $CaCl_2$ solution prior to use (*19*). For the activation of the support, 20 mL of glutaraldehyde of concentration varying from 2 to 8% (w/v) in 200 mM tris-maleic buffer at different pH values (ranging from 5.3 to 8.5) was added to 1 g of alginate beads (from 0.4 to 1.6 g of glutaraldehyde per gram of support, respectively). Support activation was carried out at 25 °C under rotary stirring (150 rpm) for 2 h. The activated beads were removed by filtration and then washed at least three times with 30 mL of distilled water to remove the glutaraldehyde, determined at 245 nm. The diameter of beads (3.4 ± 0.2 mm) was measured using an optical microscope (Zeiss) equipped with a micrometrical device.

Immobilization on Alginate−Glutaraldehyde Beads. Fifteen milliliters of enzyme solution (ranging from 10.28 to 216.53 U mL^{-1}) in 200 mM tris-maleic buffer at different pH value (5.3−8.5) was added to 25 g of activated support. The immobilization process were performed over 60 min (or at different coupling times if stated in the text) at 25 °C under rotary stirring (150 rpm). Then the beads were washed with distilled water until no enzyme activity was detected. Analyses of the protease activities carried out on the initial Neutrase solution and immobilized preparations were used to determine the activity immobilization yield (IY, %) as

$$\mathrm{IY}\ (\%) = (U_s/U_o) \times 100 \qquad (1)$$

where U_s = total activity recovered on the support and U_o = activity offered for immobilization.

Determination of Protease Activity. Soluble and immobilized Neutrase was determined according to the TCA−Lowry assay. A reaction mixture of 3 mL of 15 mg mL^{-1} casein dissolved in 100 mM tris-borate buffer (pH 8.1) and 0.6 mL of native protease—or equivalent immobilized enzyme—was incubated for 10 min at 50 °C with constant shaking at 150 rpm. The reaction was stopped by adding 1.2 mL of 17.5% (w/v) trichloroacetic acid (TCA). The precipitate was removed by filtration through Whatman no. 1 filter paper. Then, 1 mL of filtrate was mixed with 3 mL of 2% (w/v) Na_2CO_3 solution and 1 mL of 3-fold diluted Folin−Ciocalteu reagent (*20*). After vigorous mixing, the color was allowed to develop for 45 min at room temperature. The absorbance due to the amino acids produced was analyzed at 700 nm, on the basis of tyrosine as standard. One unit of activity was defined as the amount of enzyme that hydrolyzes casein to produce equivalent color to 1 μg of tyrosine per minute at pH 8.1 and 50 °C. A blank was run in the same manner, except the enzyme was added after the addition of TCA. The data presented for all protease activity determination are mean values of triplicate assays.

Statistical Methods. The immobilization parameters were optimized using response surface methodology (*21*). The central composite design was employed in this regard (*22*). Optimized conditions and response surfaces were calculated and drawn, respectively, with Statgraphics *Plus* for Windows 4.0. This software package was also used to fit the second-order model to the independent variables by using eq 2

$$y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i}^{i<j}\sum_{j} b_{ij} X_i X_j + e \qquad (2)$$

where y is the dependent variable (response variable) to be modeled, X_i and X_j are the independence variables (factors), b_0, b_i, b_{ii}, and b_{ij} are regression coefficients, and e is the error. The model was simplified by dropping terms that were not statistically significant ($p > 0.05$) by analysis of variance (ANOVA).

Determination of Kinetic Parameters. The apparent Michaelis constants (K_m) of free and immobilized Neutrase were determined by measuring the activity reaction rates (under the conditions given earlier) at substrate concentration ranging from 7.5 to 30 mg mL^{-1}. The apparent K_m value was obtained by analyzing the data according to the Hanes−Woolf equation. Turnover number (k_{cat}) and specific constant (k_{cat}/K_m) have not been calculated because Neutrase is not a pure enzyme.

Effect of pH and Temperature on Protease Activity. The effect of pH on free and immobilized protease activity was studied using three buffer solutions with pH values ranging from 4.0 to 10.0. The buffers employed in these measurements were 100 mM acetic/acetate (pH 4.0−5.5), 100 mM tris-maleate (pH 5.5−8.0), and 100 mM borate (pH 8.0−10.0).

The optimum temperature for hydrolysis of casein at pH 8.1 was determined by measuring the protease activity at seven different incubation temperatures over a range of 30−80 °C. The temperature dependence on the rate constant, for values below the temperature of inactivation, can be described by the Arrhenius equation

$$k = A \times e^{-E_a/RT} \qquad (3)$$

where k is the rate constant, A is the preexponential factor, E_a is the activation energy, R is the gas constant (8.31 J mol^{-1} K^{-1}), and T is the absolute temperature. The apparent activation energy of free and immobilized enzyme were determined from the slope of logarithmic of the activity versus the reciprocal of Kelvin temperature (slope = $-E_a/2.303$ R).

It is worth noting that, after the treatment of the immobilized enzyme at different pH values and temperatures, no protease activity was detected in the aqueous solution.

Thermal Stability. Thermal stability of both free and immobilized Neutrase was evaluated by measuring the residual activity of the enzyme exposed to various temperatures (from 30 to 90 °C) for 60 min at pH 8.1. After heating, the samples were quickly cooled and assayed for enzyme activity as described previously. The remaining activities were expresed as relative to the original activity assayed without heating.

RESULTS AND DISCUSSION

Immobilization Conditions Using Alginate−Glutaraldehyde Beads. Preliminary experiments were carried out to screen the parameters that influence the covalent immobilization of Neutrase on alginate−glutaraldehyde beads and to determine the experimental domain. From these experiments, four factors were investigated: glutaraldehyde concentration (X_1), initial enzyme concentration (X_2), immobilization pH (X_3), and contact time (X_4). The range and values of the studied variables in the first experiment are summarized in **Table 1**. A central composite design (30 runs) was chosen as a 2^4 full factorial design with star point and six supplementary trials at the center. The design was rotatable; this means that the designs have points which

Table 2. Experimental Design and Results According to the First Experiment (Central Composite Design 2^4)[a]

	variable level[b]				immobilized enzyme activity[c]	immobilization yield (%)	
run	X_1	X_2	X_3	X_4		exptl	predicted
1	−1 (2)	−1 (61.84)	−1 (6.1)	−1 (60)	4.39	12.20	9.89
2	+1 (6)	−1 (61.84)	−1 (6.1)	−1 (60)	7.79	21.00	28.30
3	−1 (2)	+1 (164.96)	−1 (6.1)	−1 (60)	7.02	7.10	10.83
4	+1 (6)	+1 (164.96)	−1 (6.1)	−1 (60)	21.26	21.50	19.62
5	−1 (2)	−1 (61.84)	+1 (7.7)	−1 (60	10.16	27.40	25.97
6	+1 (6)	−1 (61.84)	+1 (7.7)	−1 (60)	16.09	43.40	42.89
7	−1 (2)	+1 (164.96)	+1 (7.7)	−1 (60)	9.49	9.60	13.61
8	+1 (6)	+1 (164.96)	+1 (7.7)	−1 (60)	20.27	20.50	20.90
9	−1 (2)	−1 (61.84)	−1 (6.1)	+1 (160)	5.43	14.65	16.93
10	+1 (6)	−1 (61.84)	−1 (6.1)	+1 (160)	11.72	31.60	28.12
11	−1 (2)	+1 (164.96)	−1 (6.1)	+1 (160)	8.30	8.40	9.44
12	+1 (6)	+1 (164.96)	−1 (6.1)	+1 (160)	6.82	6.90	11.01
13	−1 (2)	−1 (61.84)	+1 (7.7)	+1 (160)	12.66	34.15	36.56
14	+1 (6)	−1 (61.84)	+1 (7.7)	+1 (160)	17.54	47.30	46.26
15	−1 (2)	+1 (164.96)	+1 (7.7)	+1 (160)	20.17	20.40	15.78
16	+1 (6)	+1 (164.96)	+1 (7.7)	+1 (160)	12.85	13.00	15.85
17	−α (0)	0 (113.40)	0 (6.9)	0 (110)	3.80	5.60	4.65
18	+α (8)	0 (113.40)	0 (6.9)	0 (110)	17.27	25.40	23.13
20	0 (4)	+α (216.53)	0 (6.9)	0 (110)	17.04	13.13	9.91
21	0 (4)	0 (113.40)	−α (5.3)	0 (110)	13.00	19.13	15.34
22	0 (4)	0 (113.40)	+α (8.5)	0 (110)	24.27	35.70	36.27
23	0 (4)	0 (113.40)	0 (6.9)	−α (10)	17.47	25.70	22.65
24	0 (4)	0 (113.40)	0 (6.9)	+α (210)	16.86	24.80	24.63
25	0 (4)	0 (113.40)	0 (6.9)	0 (110)	12.71	18.70	18.30
26	0 (4)	0 (113.40)	0 (6.9)	0 (110)	13.33	19.60	18.30
27	0 (4)	0 (113.40)	0 (6.9)	0 (110)	12.20	17.95	18.30
28	0 (4)	0 (113.40)	0 (6.9)	0 (110)	12.41	18.25	18.30
29	0 (4)	0 (113.40)	0 (6.9)	0 (110)	12.34	18.15	18.30
30	0 (4)	0 (113.40)	0 (6.9)	0 (110)	11.66	17.15	18.30

[a] Run 19 was considered to be an outlier and removed from the design. [b] Numbers in parentheses represent actual experimental amounts. X_1, X_2, X_3, and X_4 were glutaraldehyde concentration (%, w/v), enzyme loading (U mL^{-1}), immobilization pH, and contact time (min), respectively. [c] In U g^{-1} of support. Immobilization conditions: 0.6 mL of enzyme solution per gram of support.

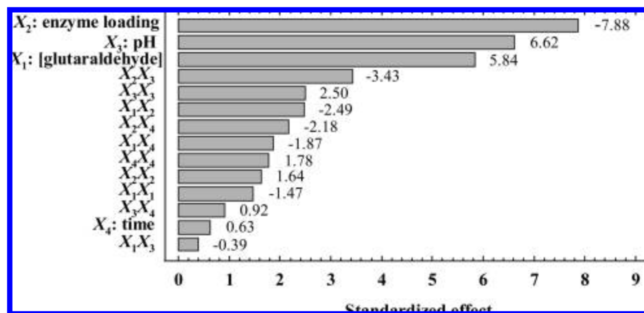

Figure 1. Pareto chart of standardized effect for the first experiment (central composite design 2^4).

are equidistant from the center. Experiments at the center were carried out to obtain an estimation of the experimental error.

Table 2 shows the designed experiment matrix, together with the experimental results. The immobilization yield varied strongly (from 5.60 to 47.3%). High percentages of immobilization (>40%) were reached when a low level of Neutrase loading was used at maximun level of both glutaraldehyde concentration and immobilization pH (runs 6 and 14). As can be noted, the support activated with glutaraldehyde exerted a positive influence on immobilization yield (runs 17 and 18). This suggests that glutaraldehyde is a suitable activation agent for Neutrase immobilization on alginate beads. The inclusion of a spacer, glutaraldehyde, was essential to improve conformational flexibility (*23*) and enhance enzymatic activity in comparison with the immobilized enzyme without this bifunctional reagent.

Table 3. Independent Variables and Their Levels Used for the Second Experiment (Central Composite Design 2^3)[a]

		coded variable levels				
independent variable	symbol	−α	−1	0	+1	+α
glutaraldehyde (%, w/v)	X_1	4.32	5.00	6.00	7.00	7.68
enzyme loading (U mL^{-1})[b]	X_2	20.56	41.12	61.84	82.40	102.96
immobilization pH	X_3	5.24	5.55	6.00	6.45	6.76

[a] Contact time: 60 min. [b] Immobilization condition: 0.6 mL of enzyme solution per gram of support.

Table 4. Experimental Design and Results According to the Second Experiment (Central Composite Design 2^3)[a]

	variable level[b]			immobilized enzyme activity[c]	immobilization yield (%)	
run	X_1	X_2	X_3		exptl	predicted
1	−1 (5.00)	−1 (41.12)	−1 (5.55)	14.50	58.63	60.67
2	+1 (7.00)	−1 (41.12)	−1 (5.55)	16.44	66.51	67.18
3	−1 (5.00)	+1 (82.40)	−1 (5.55)	9.97	20.16	14.36
4	+1 (7.00)	+1 (82.40)	−1 (5.55)	12.55	25.39	23.22
5	−1 (5.00)	−1 (41.12)	+1 (6.45)	15.58	63.00	64.63
6	+1 (7.00)	−1 (41.12)	+1 (6.45)	14.21	57.48	62.75
7	−1 (5.00)	+1 (82.40)	+1 (6.45)	18.29	37.00	35.79
8	+1 (7.00)	+1 (82.40)	+1 (6.45)	19.20	38.83	36.25
9	−α (4.32)	0 (61.84)	0 (6.00)	9.07	24.45	26.17
10	+α (7.68)	0 (61.84)	0 (6.00)	12.23	33.00	32.03
11	0 (6.00)	−α (20.56)	0 (6.00)	13.29	107.52	101.54
12	0 (6.00)	+α (102.96)	0 (6.00)	20.76	33.59	40.32
13	0 (6.00)	0 (61.84)	−α (5.24)	10.28	27.74	30.61
14	0 (6.00)	0 (61.84)	+α (6.76)	17.43	47.01	44.90
15	0 (6.00)	0 (61.84)	0 (6.00)	19.54	52.69	51.21
16	0 (6.00)	0 (61.84)	0 (6.00)	18.70	50.42	51.21
17	0 (6.00)	0 (61.84)	0 (6.00)	18.85	50.83	51.21
18	0 (6.00)	0 (61.84)	0 (6.00)	18.56	50.06	51.21
19	0 (6.00)	0 (61.84)	0 (6.00)	18.61	50.20	51.21
20	0 (6.00)	0 (61.84)	0 (6.00)	19.72	53.18	51.21

[a] Contact time: 60 min. [b] Numbers in parentheses represent actual experimental amounts. X_1, X_2, and X_3 were glutaraldehyde concentration (%, w/v), enzyme loading (U mL^{-1}), and immobilization pH, respectively. [c] In U g^{-1} of support. Immobilization conditions: 0.6 mL of enzyme solution per gram of support.

Figure 1 shows the graphical representation (Pareto plot) of the "size effect" of each of the parameters investigated upon immobilization yield. In this treatment a parameter is deemed to have a significant influence if the size effect is >2. The analysis of the overall data set indicated that contact time between the enzyme and support (X_4) was not a significant factor, whereas X_2, X_3, and X_1 showed the most pronounced effect on the response (**Figure 1**). Therefore, the immobilization time was fixed to 60 min, and the factors glutaraldehyde concentration (X_1), enzyme loading (X_2), and immobilization pH (X_3) were assessed in a second experiment. Supports activated with glutaraldehyde allowed the immobilization of proteins even at acidic−neutral pH values, suggesting that the immobilization may involve an exposed residue with a significant reactivity even at these pH values (e.g., the terminal amino groups of proteins). At higher pH values, where the activity of Lys may be significant, these groups become inactivated. Therefore, the immobilization of proteins at acidic−neutral pH values on this type of support may occur mainly via the most reactive and exposed amine groups (very likely, the terminal amino group) (*24*). Furthermore, it has also been demonstrated that alkaline pH and high glutaraldehyde concentration yield an uncontrolled reaction that generates the polymerization of glutaraldehyde solution (*25*). To develop a stable biocatalyst, a lower pH than the first design model predicted (about pH 8.0) was chosen. The values of the experimental factors are detailed

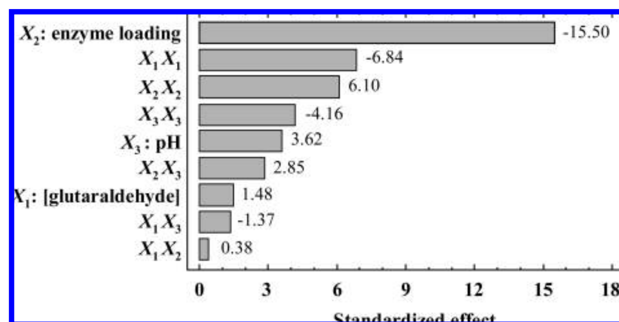

Figure 2. Pareto chart of standardized effect for the second experiment (central composite design 2^3).

Table 5. Analysis of Variance (ANOVA) for Response Surface Quadratic Model for the Immobilization of Neutrase on Alginate−Glutaraldehyde Beads[a]

source	SS	df	MS	*F* value	*p* value
X_1: [glutaraldehyde]	41.47	1	41.47	2.20	0.1686
X_2: enzyme loading	4524.43	1	4524.43	240.32	0.0000
X_3: pH	246.56	1	246.56	13.10	0.0047
X_1X_1	880.34	1	880.34	46.76	0.0000
X_1X_2	2.73	1	2.76	0.16	0.7098
X_1X_3	35.28	1	35.28	1.87	0.2010
X_2X_2	700.78	1	700.78	37.22	0.0001
X_2X_3	152.6	1	152.6	8.11	0.0173
X_3X_3	326.19	1	326.19	17.33	0.0019
total			8.68		

[a] SS, sum of squares; df, degrees of freedom; MS, mean square. $R^2 = 0.9741$; $R^2_{adj} = 0.9508$; CV = 4.34%.

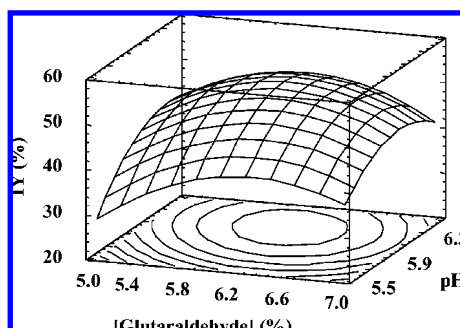

Figure 3. Response surface plot for immobilization yield as a function of immobilization pH and glutaraldehyde concentration (enzyme loading = 61.84 U mL^{-1}).

in **Table 3**. A central composite design (20 runs) was chosen as a 2^3 full factorial design with star point and six extra center points. The design of experiments and respective experimental yields are given in **Table 4**. The results showed that alginate binding capacity was strongly affected by initial enzyme protease concentration. The immobilization yield reached its maximum value (107%) at 20.56 U mL^{-1} of enzyme loading (run 11).

The analysis of the overall data set indicated that enzyme concentration had the most pronounced effects on response, although the immobilization pH exerted a statistically significant effect, as did the quadratic effects and the interaction X_2X_3 (**Figure 2**). In fact, the enzyme concentration had a negative effect, meaning that its decrease maximizes the overall response. Despite this, low enzyme loading led to biocatalyst with low activity (**Table 4**). Therefore, it was decided to set 61.84 U mL^{-1} of Neutrase for determining the optimal conditions of immobilization.

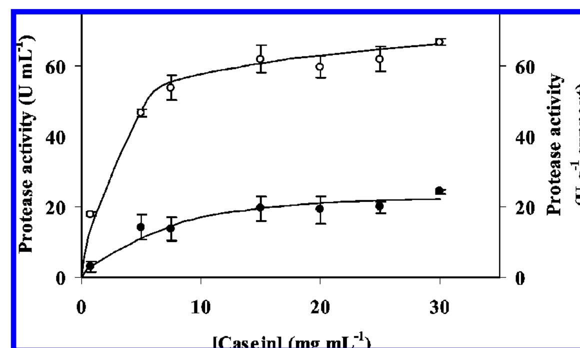

Figure 4. Michaelis−Menten kinetics for Neutrase free (○) and immobilized on alginate−glutaraldehyde beads (●).

Analysis of variance (ANOVA) was important in determining the adequacy and significance of the quadratic model. ANOVA summary is shown in **Table 5**. The fitness of the model was expressed by the R^2 value, which is 0.9741, indicating that 97.41% of the variability in the response can be explained by the model. The adjusted R^2 value of 0.9508 suggested that the model was significant. A very low value of coefficient of the variation (CV = 4.34%) clearly indicated a very high degree of precision and a good deal of reliability of the experimental values.

A central composite design provides sufficient data for the fitting of a second-degree expression. In this sense, eq 4 explains the data obtained in the second experiment.

$$\mathrm{IY} = -1.01\times10^3 - 5.90X_2 + 2.90\times10^2X_3 - 7.82(X_1)^2 + 0.02(X_2)^2 + 0.47X_2X_3 - 2.35\times10^1(X_3)^2 \quad (4)$$

X_1, X_2, and X_3 are coded values for glutaraldehyde concentration, Neutrase loading, and immobilization pH, respectively.

The immobilization conditions maximized the response by keeping enzyme concentration at its minimum. The effect of the glutaraldehyde concentration and pH on the response at a fixed enzyme loading of 61.84 U mL^{-1} is illustrated in **Figure 3**. The immobilization efficiency was remarkably low at low values of pH and glutaraldehyde concentration. Increase in both pH and glutaraldehyde concentration yielded an increase in the response surface. The response value reached its highest level at 6.2% (w/v) glutaraldehyde, whereas pH showed a maximum at pH 6.2. Under these conditions, the model predicted an immobilization yield of 52.18%.

To confirm this result, a validation assay was conducted in the conditions imposed as the optimum. In this assay an immobilization yield of 51% was obtained. This value is in good agreement with the predicted values for the analyzed response, validating the mathematical models attained in the studied region.

As expected, the immobilization enzyme presented activity values lower than those obtained for the free enzyme. The decrease in activity of the immobilized enzyme could be considered to be due to diffusional limits, steric effects, structural changes in the enzyme ocurring upon covalent coupling, or lowered accesibility of substrate to the active site of the immobilized enzyme (*26, 27*).

Determination of Kinetic Parameters in Soluble and Immobilized Enzyme. The catalytic activity of free and immobilized Neutrase was assessed using casein as the sustrate (**Figure 4**). It seems that both free and immobilized enzyme followed Michaelis−Menten kinetics. The value of kinetic parameter K_m was obtained by the Hanes−Woolf plot. The linear regression analysis indicated that quality of fit was quite good,

Table 6. Enzymatic Properties of Neutrase Free and Immobilized on Alginate−Glutaraldehyde Beads

property	Neutrase	
	free	immobilized
K_m (mg mL^{-1})	2.4 ± 0.1	6.4 ± 0.2
optimun pH	8.0	5.5
optimun temperature (°C)	50	60
E_a (kJ mol^{-1})	42.7 ± 0.3	22.0 ± 0.3

with R^2 values of 0.99 and 0.96 for free and immobilized enzyme, respectively. The apparent K_m value for free enzyme was determined to be 2.4 mg mL^{-1} (**Table 6**). It appears that the immobilized protease has a Michaelis constant higher than that of the soluble enzyme (6.4 mg mL^{-1}). An increase in K_m once an enzyme has been immobilized indicates that the immobilized enzyme has an apparently lower affinity for its substrate than that of the native enzyme, which may be caused by the steric hindrance of the active site by the support or the loss of enzyme flexibility necessary for substrate binding (*26*).

Effect of pH and Temperature on Enzyme Activity. The performance of immobilized enzymes was studied by changing the pH of the medium. pH is one of the most important factors influencing not only the side groups of the amino acid dissociations in the protein structure but also the solution chemistry of the insoluble support. Thus, protein−support interaction and surface properties of a protein are strongly influenced by the pH of the solution.

Figure 5 shows that the pH range at which the immobilized enzyme had high activity (>50%) was considerably widened compared to that of the free one, probably due to diffusional limitations of the immobilized enzyme molecules. Similar behavior has been described for the immobilization of papain on poly(vinyl alcohol) beads activated by hexamethylene diisocyanate (*28*). Additionally, a very significant activity change for free enzyme, at pH 8, was observed depending on buffer composition (**Figure 5A**). This effect of buffer was also described by Jeohn et al. (*29*) for a metalloendopeptidase, obtaining about 30% of activity increase by varying the buffer. Perrin and Dempsey (*30*) suggested that buffer composition can affect enzyme activity in different ways: ionic strength, interaction with enzyme conformation or active site; interaction with substrate, inhibitors, or cofactors and/or complexing with metals. Similar behavior but in lower extent (**Figure 5B**) was observed for immobilized enzyme, probably because immobilization could provide the enzyme some protection against buffer composition.

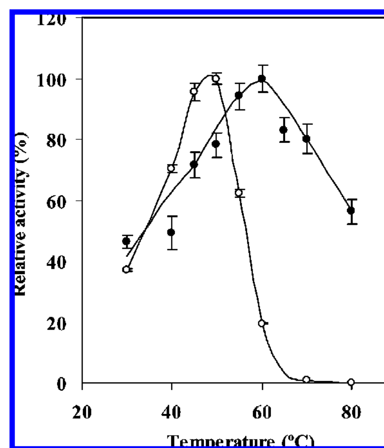

Figure 6. Effect of temperature on the activity of Neutrase free (○) and immobilized on alginate−glutaraldehyde beads (●). Relative activity was expressed as a percentage of maximum activity under experimental conditions.

The immobilization may permit a reduction of the inhibition problems by different mechanisms: (i) exclusion of the inhibitor from the enzyme environment and (ii) decrease of the affinity of the recognition places of the enzyme by the inhibitor (*31*).

The effect of temperature on the activity of free and immobilized Neutrase was determined in the temperature range of 30−80 °C. As given in **Figure 6**, optimum temperature recorded was at 50 °C for free enzyme and at 60 °C for immobilized enzyme. The increase in the optimum temperature may be because of the improvement in the enzyme rigidity upon immobilization by covalent binding. Similar displacement of optimun temperature for immobilized enzymes was observed in many cases, but the extent of displacement differed from matrix to matrix and with the kind of interaction between the enzyme and matrix. For example, a displacement of optimum temperature from 40 to 50 °C was observed for alkaline protease from *Conidiobolus macrosporus* immobilized on polyamide using glutaraldehyde as bifunctional agent (*32*). Rao et al. (*33*) suggested that the shift in optimal temperature toward higher temperature might be due to the immobilization of the enzyme to the support, providing stability and resulting in formation of the enzyme−substrate complex without any hindrance for the access of substrates to the active site.

Furthermore, the apparent activation energies for free and immobilized Neutrase were also evaluated and are shown in **Table 6**. The activity of the immobilized enzyme was less affected by temperature than was the free enzyme, because

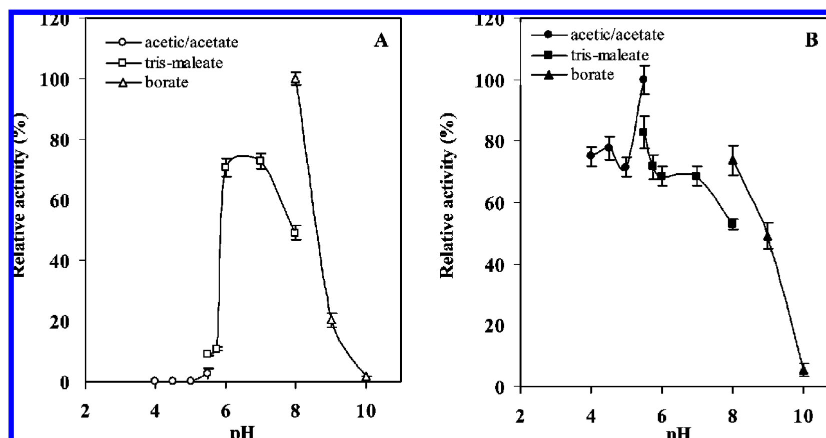

Figure 5. Effect of pH on the activity of Neutrase free (**A**) and immobilized on alginate−glutaraldehyde beads (**B**). Relative activity was expressed as a percentage of maximum activity under experimental conditions.

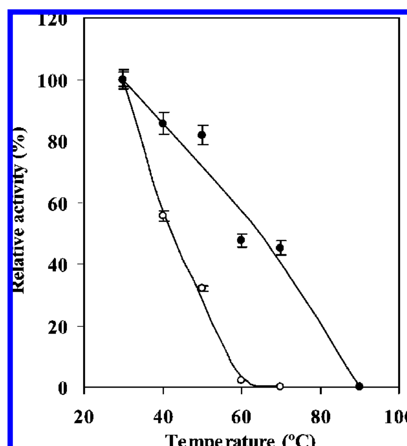

Figure 7. Thermal stability of Neutrase free (○) and immobilized on alginate−glutaraldehyde beads (●). The enzyme was preincubated during 1 h at the indicated temperatures. Relative activity was expressed as a percentage of the original activity assayed without heating.

immobilization lowered the activation energy (from 42.7 to 22.0 kJ mol^{-1}), resulting in a higher catalytic efficiency for immobilized Neutrase. The observed decrease in the activation energy confirmed that there was mass-transfer control for the immobilized enzyme rather than kinetic control. Similar results have been described for the immobilization of pepsin (*34*) and neutral proteases (*35*) on chitosaneous supports activated by glutaraldehyde.

Thermal Stability. Neutrase immobilized on alginate−glutaraldehyde beads was heated for 1 h at different temperatures to measure the thermal stability, and the results of residual activity are shown in **Figure 7**. The covalent immobilization of the enzyme to the support caused an increase of thermal stability. The free and immobilized enzymes heated at 50 °C showed 79 and 18% reductions in activity, respectively, and at 70 °C the immobilized retained 45% of initial activity, whereas free enzyme was completely inactivated after the same treatment. The higher stability of the immobilized protease could be due to the diminished autoproteolysis of the enzyme fixed to the support. The autoproteolysis of proteases in aqueous solutions may significantly inactivate the enzyme. The restricted interaction among the alginate−glutaraldehyde immobilized enzyme molecules could play an important role in retaining the enzyme activity (*36*). On the other hand, various authors (*37, 38*), with regard to enzyme stability, generally assume unfolding of the protein structure to be one of the main phenomena involving the irreversible mechanism in enzyme inactivation. In such cases, it may be supposed that the stability results are closely connected to variations in the conformational structure of protease and particularly in the rigidity of the secondary and tertiary structure of enzyme that reduces the unfolding rate. Now, the relative distance among all residues involed in the multipoint immobilization have to be maintained unaltered during any conformational change induced by any distorting agent. This should reduce any conformational change involved in enzyme inactivation and greatly increase the enzyme statility (*31, 39, 40*).

In conclusion, Neutrase immobilization could be carried out successfully using as support alginate−glutaraldehyde beads. The efficiency of immobilization achieved was about 50%. The protease immobilized had a broader pH profile, indicating the effectiveness of support in providing resistance to wide variation in pH. Moreover, enzyme−support covalent attachment proved to enhance the temperature optimum and thermal stability of the immobilized enzyme compared to the soluble one. Immobilization also eliminates the need for an inactivation process because the immobilized enzyme can be easily removed by filtration, facilitating a better control of the hydrolytic process and avoiding contamination with the catalyst in the production of protein hydrolysates.

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