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BIOACTIVE POLYMERS. XLVI. IMMOBILIZATION OF PEPSIN ON *N*-(4-CARBOXYPHENYLCARBAMOYLMETHYL)-CELLULOSE

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ABSTRACT

The immobilization of pepsin on *N*-(carboxyphenylcarbamoylmethyl)-cellulose with dicyclohexylcarbodiimide as activator is studied. The coupling reaction is influenced by enzyme/support and activator/support ratios, pH, and duration. The Michaelis constant for the immobilized enzyme is lower than that for the free one.

INTRODUCTION

The immobilization of pepsin on both organic and inorganic synthetic polymers has been widely investigated [1-5]. Natural polymers and their derivatives are advantageous due to their pronounced hydrophilicity and conformations permitting the formation of microsystems favorable to enzyme activity [6, 7].

In the present paper, pepsin immobilization on *N*-(4-carboxyphenylcarbamoylmethyl)cellulose (N-CPhCMC) is reported. This polymer is used for the first time as a support for enzyme immobilization. It is characterized by higher reactivity (due to carboxylic groups bonded to aromatic rings) and reduced swelling in aqueous media.

EXPERIMENTAL

N-CPhCMC was synthesized in two stages, starting from carboxymethylcellulose (CMC) with a substitution degree DS = 0.78 and viscosity-average molecular weight $\bar{M}_v$ = 124 000. In the first stage, (chlorocarbonylmethyl)-cellulose (CMC-Cl) [8] was obtained and then condensed with *p*-aminobenzoic acid, leading to N-CPhCMC [9]. The support polymer had a substitution degree with $-C_6H_4-COOH$ groups of 0.31.

The necessary amount of pepsin was dissolved under stirring in 10 mL buffer of different pH values, according to the experimental design (Table 1); 0.1 g N-CPhCMC was separately swollen in 10 mL buffer under stirring for 30 min, and then the pepsin solution was added in small portions. Finally, a solution of dicyclohexylcarbodiimide (DCI) (in amounts according to the experimental design) in 2 mL tetrahydrofuran (THF) was added when the reaction began. The mixture was maintained under good stirring at 5 ± 1°C for the time required by the experimental design. When the reaction was over, 10 mL acetone was added to remove the unreacted pepsin, DCI, and dicyclohexylurea formed, the product being separated by centrifugation. It was then washed under stirring with 20 mL buffer of pH 2 (0.2 *M* KCl and 0.02 *M* HCl), centrifuged (15 min, 4000 rpm), these operations being repeated three times until no traces of pepsin were detected in the supernatant layer (Lowry method [10]).

The insoluble enzymatic product was finally suspended in a buffer solution of pH 5 and kept at 4°C.

The activity of the coupled enzyme was estimated by the modified Anson method [11].

EXPERIMENTAL DESIGN

The coupling of pepsin on different natural macromolecular supports is influenced by several factors, the most important being the pepsin/support (g/g) ratio, pH, the activator/support (g/g) ratio, and the reaction time [12].

In order to correlate the activity of the products with the reaction parameters, a regression equation of the following type was proposed:

$$Y = a_0 + \sum a_i x_i + \sum a_{ij} x_i x_j, \quad i \leqslant j, \tag{1}$$

where Y is the enzymatic activity; a_0, a_i, and a_{ij} are regression coefficients; and x_i and x_j are the independent variables.

A great number of experiments are necessary in order to establish the values of the regression coefficients. We carried these experiments out according to a centrate, rotatable, composed, second-order experimental design, which implies three groups of experiments [13]: factorial ones, additional ones for the second-order equations, and central ones to determine the experimental errors.

The number of experiments was 31, 7 of them being in the center of experimental domain (Table 1).

This experimental design has the following advantages: 1) the experiments are equally and uniformly distributed in the whole experimental domain, consequently the function obtained is also well-defined in the whole domain; 2) the mathematical model obtained is more precise; 3) because the number of experiments is reduced, the work volume and the necessary materials are also reduced, and the computation of regression coefficients is simpler.

The standard error is the same for all points at the same distance (ρ) from the center of the experimental domain, and satisfies the relation

$$\rho = \sqrt{\sum x_i^2} = \text{constant.} \tag{2}$$

Each variable is taken at five levels (Table 1), and coded according to the following relation to simplify the computations:

$$x_i = \frac{X_i - X_{ic}}{\Delta X_i}, \tag{3}$$

where x_i is the coded variable, X_i is the actual variable, X_{ic} is the value of the actual variable at the center of the experimental domain, and ΔX_i is the range of X_i.

To compute the regression coefficients, the multiple regression method was applied, based (for 4 independent variables) on the following relations:

$$\begin{aligned} a_0 &= 0.142857 \sum Y - 0.035714 \sum\sum x_i^2 Y, \\ a_i &= 0.041667 \sum x_i Y, \\ a_{ii} &= 0.031250 \sum x_i^2 Y + 0.003720 \sum\sum x_i^2 Y - 0.035714 \sum Y, \\ a_{ij} &= 0.0625 \sum x_i x_j Y. \end{aligned} \tag{4}$$

TABLE 1. Experimental Design and Results

Variable								
Pepsin/N-CPhCMC		pH		DCI/N-CPhCMC		Duration		Activity
x_1	Actual, g/g	x_2	Actual	x_3	Actual, g/g	x_4	Actual, h	(μmol·g^{-1}·min^{-1})
-1	0.150	-1	2.5	-1	0.45	-1	11	0.953
1	0.400	-1	2.5	-1	0.45	-1	11	1.506
-1	0.150	1	5.5	-1	0.45	-1	11	0.106
1	0.400	1	5.5	-1	0.45	-1	11	0.135
-1	0.150	-1	2.5	1	0.95	-1	11	0.073
1	0.400	-1	2.5	1	0.95	-1	11	0.095
-1	0.150	1	5.5	1	0.95	-1	11	0.013
1	0.400	1	5.5	1	0.95	-1	11	0.038
-1	0.150	-1	2.5	-1	0.45	1	25	3.083
1	0.400	-1	2.5	-1	0.45	1	25	5.039
-1	0.150	1	5.5	-1	0.45	1	25	0.989
1	0.400	1	5.5	-1	0.45	1	25	1.256
-1	0.150	-1	2.5	1	0.95	1	25	1.443
1	0.400	-1	2.5	1	0.95	1	25	2.742

-1	0.150	1	5.5	1	0.95	1	25	0.482
1	0.400	1	5.5	1	0.95	1	25	0.786
-2	0.025	0	4.0	0	0.70	0	18	0.696
2	0.525	0	4.0	0	0.70	0	18	2.485
0	0.275	-2	1.0	0	0.70	0	18	1.033
0	0.275	2	7.0	0	0.70	0	18	0.933
0	0.275	0	4.0	-2	0.20	0	18	3.092
0	0.275	0	4.0	2	1.20	0	18	1.247
0	0.275	0	4.0	0	0.70	-2	4	0.897
0	0.275	0	4.0	0	0.70	2	32	2.361
0	0.275	0	4.0	0	0.70	0	18	1.649
0	0.275	0	4.0	0	0.70	0	18	1.361
0	0.275	0	4.0	0	0.70	0	18	1.311
0	0.275	0	4.0	0	0.70	0	18	1.394
0	0.275	0	4.0	0	0.70	0	18	1.599
0	0.275	0	4.0	0	0.70	0	18	1.298
0	0.275	0	4.0	0	0.70	0	18	1.467

The standard deviation s of Y for the values in the center of the experimental domain ($n = 7$) was calculated by the relation

$$s = \sqrt{\frac{\Sigma(Y - \bar{Y})^2}{n - 1}}, \tag{5}$$

where $\bar{Y}$ is the average value in the center of the experimental domain. The value of s from the seven last Y values in Table 1 was 0.138.

The standard errors of the regression coefficients are computed from s as follows:

$$\begin{aligned} s_{a_i} &= 0.204s = 0.028, \\ S_{a_{ii}} &= 0.185s = 0.026, \\ S_{a_{ij}} &= 0.250s = 0.035. \end{aligned} \tag{6}$$

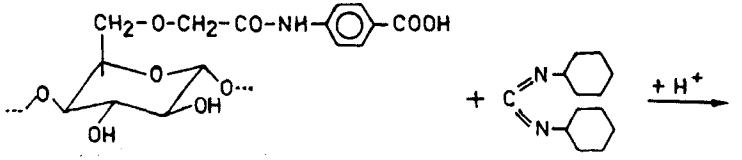
The regression equation (obtained on a FELIX X 256 computer) is

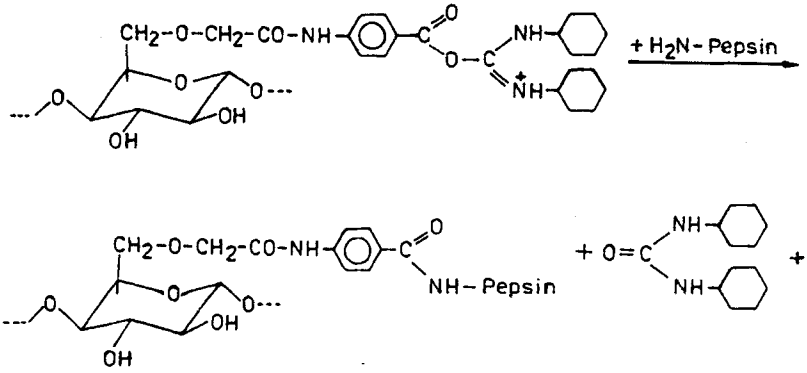
$$\begin{aligned} A = {} & 1.435 + 0.337x_1 - 0.47x_2 - 0.46x_3 + 0.658x_4 - 0.032x_1^2 \\ & - 0.203x_1x_2 - 0.184x_1x_3 - 0.075x_1x_4 + 0.314x_2^2 + 0.113x_2x_3 \\ & + 0.203x_2x_4 - 0.401x_3^2 - 0.15x_3x_4 - 0.022x_4^2. \end{aligned}$$

The high values of the multiple correlation coefficient (0.927) and of the F factor (7.334) indicate that the derived equation provides a good description of the dependence of the activity on the parameters under study.

RESULTS AND DISCUSSION

The coupling of pepsin to N-CPhCMC is based on the reaction of amino groups of the enzyme with the carboxy ones on the support, in the presence of DCI as activating agent, in an acid medium:





The influence of the coupling parameters on the coupling reaction was illustrated by choosing three variables (in the center of the experimental domain or at any point) which give simple relationships. They correlate the activity of the obtained enzymatic products with the fourth variable. The results, illustrated in Figs. 1-4, all depend on the regression equation and are subject to its inherent errors.

The amount of coupled enzyme is influenced significantly by the pepsin/N-CPhCMC ratio (Fig. 1). The activity of enzymatic products is seen to increase continuously with the amount of enzyme in the initial mixture, for every reaction time, without any maximum within the experimental domain. Higher activities are obtained with longer reaction times.

The pH of the medium also affects the coupling of enzyme (Fig. 2). An increase in pH results in a decrease of enzymatic activity due to the pepsin denaturation. Thus, the optimum value of this parameter might be estimated to be about 1.5, shifting to higher values with increasing activator amount in the reaction mixture.

The activity is also influenced strongly by the DCI/N-CPhCMC ratio (Fig. 3). Higher values of activity are obtained with low amounts of DCI activator. An increase in DCI amount promotes inter- and intramolecular condensations of both coupled and free enzyme, leading to its denaturation. At the DCI/N-CPhCMC ratio of 0.2, all carboxylic groups on the support are activated, the catalytic effect of intramolecular condensation being thus limited [14].

The activity of the coupling products is seen to increase continuously with the reaction time (Fig. 4), showing the highest values at a low activator/support ratio.

The efficiency of the coupling reaction is strongly influenced by the correlated actions of the parameters under study. Thus, Figs. 5 and 6 give addi-

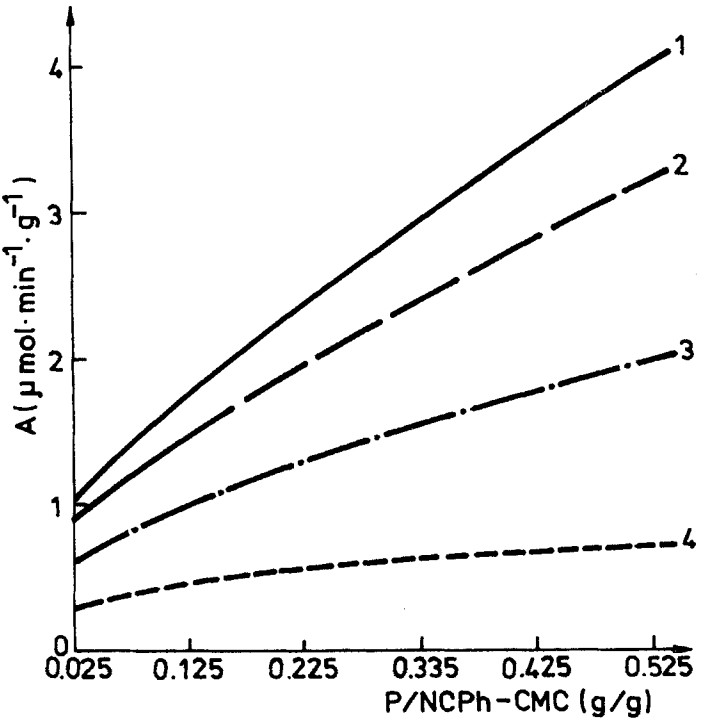


FIG. 1. Influence of pepsin/N-CPhCMC ratio on enzymatic activity of the coupling products. Reaction time: (1) 32 h; (2) 26.4 h; (3) 18 h; (4) 9.6 h. pH = 4; DCI/N-CPhCMC = 0.7 g/g.

tional information on the reaction conditions that lead to products of maximum enzymatic activity. The curves of constant activity show the highest activity to be obtained with high enzyme/support ratio and low pH (Fig. 5).

The efficiency of coupling is also increased by low amounts of activator and long reaction times (Fig. 6).

Although no absolute optimum was obtained in the chosen experimental domain, the results shown above indicate the most favorable conditions of the coupling reaction to be at about pepsin/N-CPhCMC = 0.425 g/g, pH = 2.5, DCI/N-CPhCMC = 0.2 g/g, and duration = 25 h.

A synthesis carried out under these conditions resulted in an enzymatic product with an activity of 5.11 $\mu mol \cdot g^{-1} \cdot min^{-1}$ being obtained.

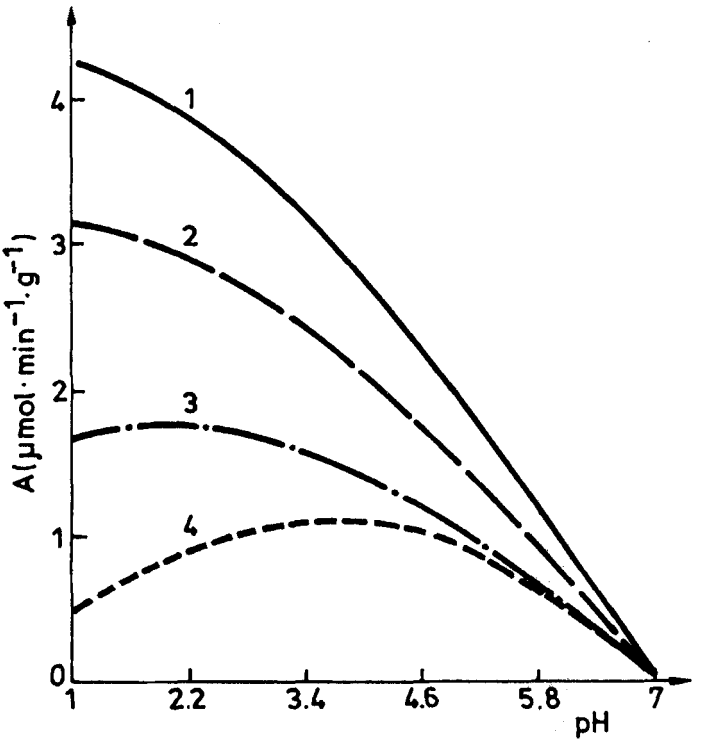


FIG. 2. Influence of pH on enzymatic activity of the coupling products. DCI/N-CPhCMC: (1) 0.2 g/g; (2) 0.4 g/g; (3) 0.7 g/g; (4) 1 g/g. Pepsin/N-CPhCMC = 0.275 g/g; reaction time = 18 h.

In order to obtain information on the kinetics of the process catalyzed by pepsin, the Michaelis-Menten constant was calculated for both free and immobilized enzyme under the above conditions. The simplified equation describing the kinetics of the enzymatic process has the form

$$v = \frac{V_m [S]}{K_m + [S]}, \tag{7}$$

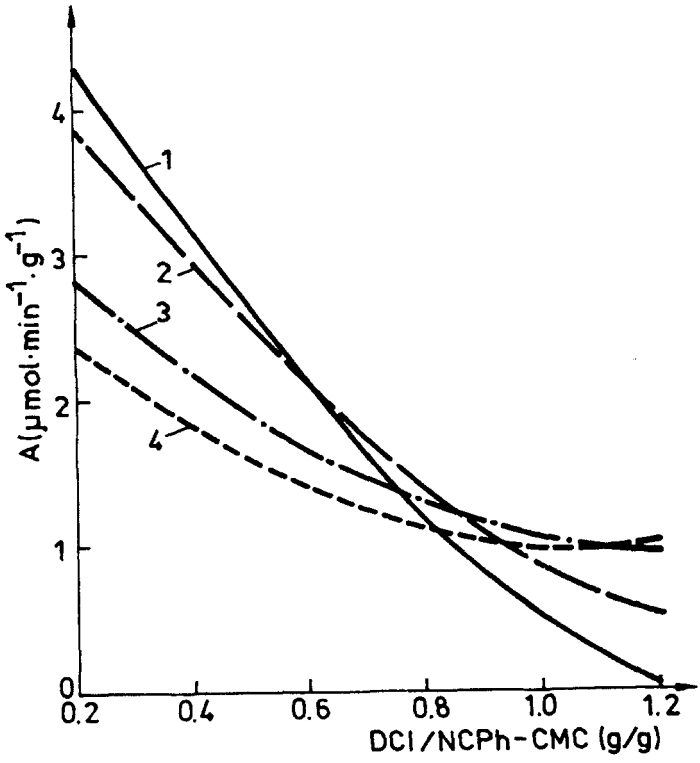


FIG. 3. Influence of DCI/N-CPhCMC ratio on enzymatic activity of the coupling products. pH: (1) 1; (2) 2.2; (3) 4; (4) 4.6. Pepsin/N-CPhCMC = 0.275 g/g; reaction time = 18 h.

where v is the reaction rate, V_m is the maximum reaction rate, [S] is the substrate concentration, and K_m is the Michaelis-Menten constant.

The K_m constant was computed from Lineweaver-Burk plots, based on the following form of Eq. (7):

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{[S]}. \tag{8}$$

The plots are shown in Figs. 7 and 8 [16].

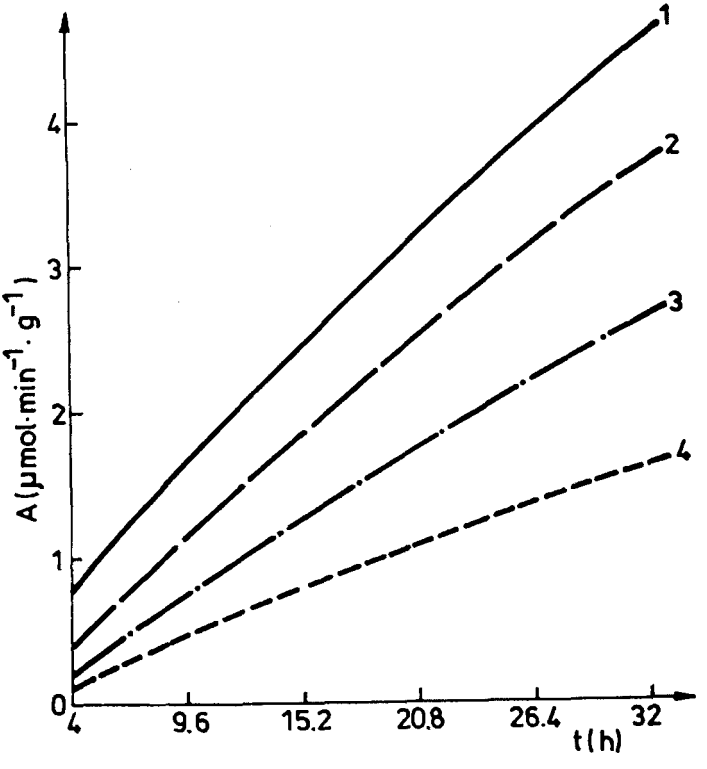


FIG. 4. Influence of reaction time on enzymatic activity of the coupling products. DCI/N-CPhCMC: (1) 0.2 g/g; (2) 0.4 g/g; (3) 0.7 g/g; (4) 1.4 g/g. Pepsin/N-CPhCMC = 0.275 g/g; pH = 4.

The Michaelis constant is seen to be smaller for the immobilized enzyme (K_m = 1.98 mg/mL for free enzyme and K_m = 1.69 mg/mL for coupled enzyme), which indicates strong electrostatic interactions between the support (polyanion) and the protein chain [15].

Some polymer-substrate (hemoglobin) interactions may also exist which would increase the concentration of the latter near the active center of enzyme [14].

Moreover, N-CPhCMC was found to give lower enzymatic activity than Biozan R [12]. Although the functional groups are more reactive for

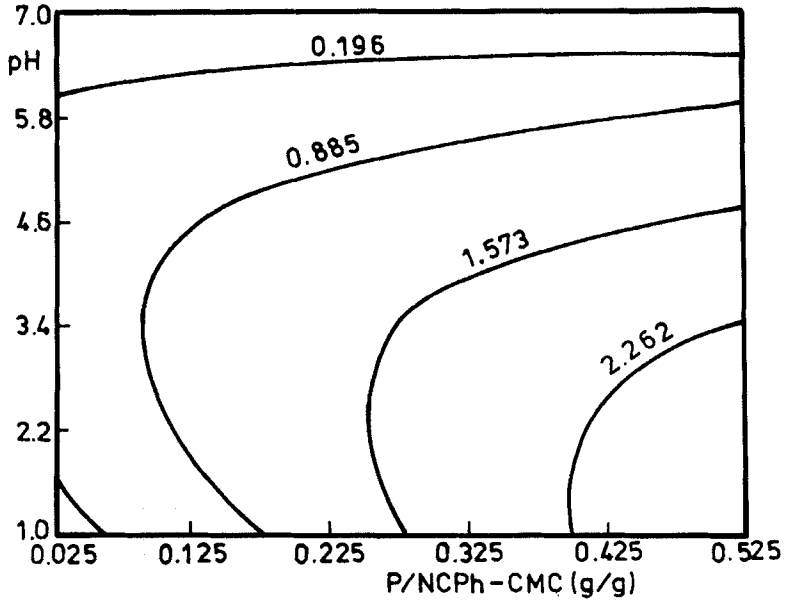


FIG. 5. Constant-activity curves in the pepsin/N-CPhCMC-pH experimental plane. DCI/N-CPhCMC = 0.7 g/g; reaction time = 18 h.

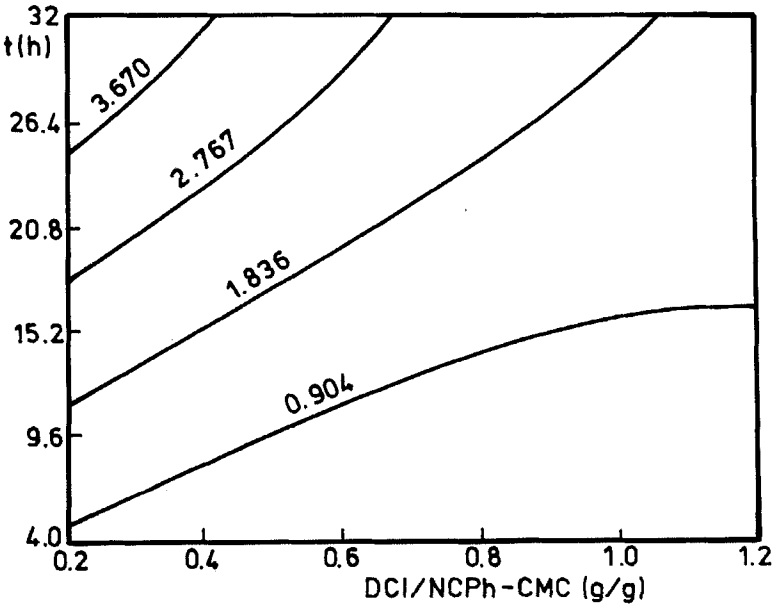


FIG. 6. Constant-activity curves in the DCI/N-CPhCMC-duration experimental plane. Pepsin/N-CPhCMC = 0.275 g/g; pH = 4.

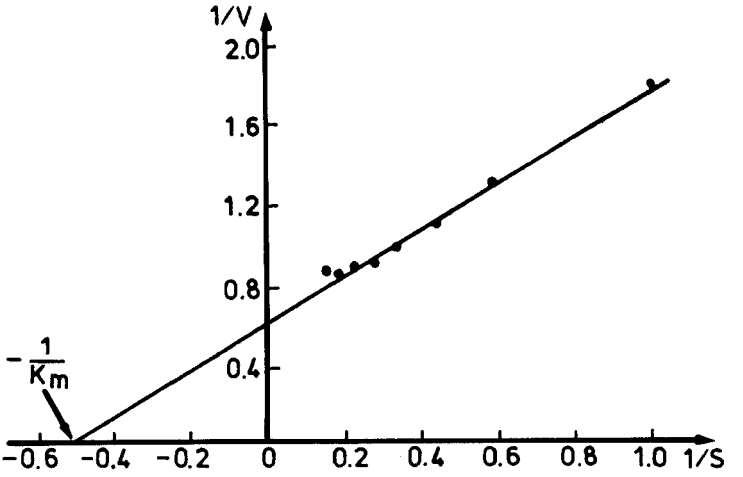


FIG. 7. Lineweaver-Burk plot for free enzyme.

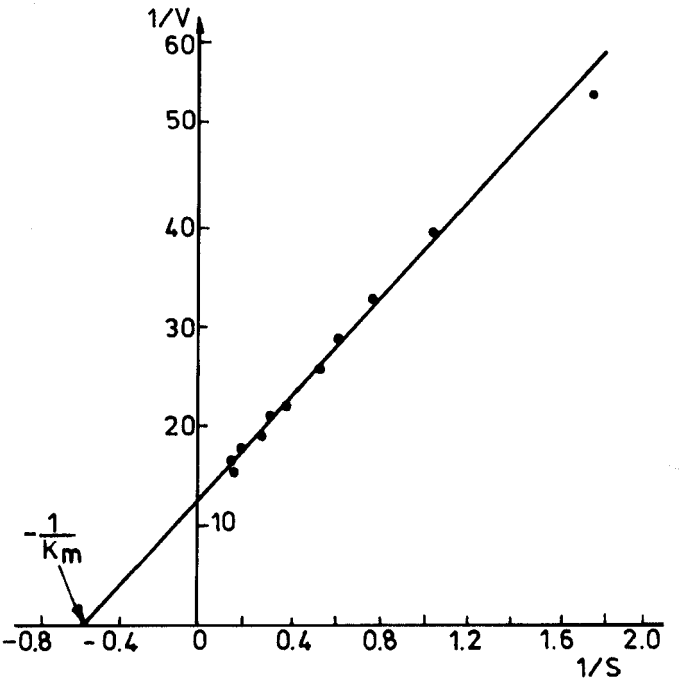


FIG. 8. Lineweaver-Burk plot for pepsin coupled to N-CPhCMC.

N-CPhCMC, the amount of coupled enzyme is lower. This is explained by the lower swelling in water of N-CPhCMC compared to Biozan R; as a consequence, access of the activator and enzyme to the carboxylic groups of the support is reduced.

On the other hand, the insolubility of the immobilized enzymatic samples is advantageous, permitting their utilization in columns for continuous enzymatic processes.

Preliminary tests also proved the possibility of using immobilized pepsin on N-CPhCMC for a minimum of three hydrolytic cycles, after which the enzymatic activity was reduced by ~25% compared to the initial one.

CONCLUSIONS

1. Pepsin may be immobilized by covalent bonding on *N*-(4-carboxyphenylcarbamoylmethyl)cellulose, with DCI as an activator, to give an insoluble product with high catalytic activity.
2. The coupling of pepsin on N-CPhCMC depends on several parameters: enzyme/support and activator/support ratios, pH, reaction time.
3. Products of high catalytic activity are obtained at high enzyme support ratio, low pH, long reaction time, and low activator/support ratio.
4. The Michaelis constant corresponding to the coupled enzyme is lower than that for the free enzyme.

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