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Cristofor Simionescu[a]; Severian Dumitriu[a]; Marcel Popa[a]; Cristina Coman[a]; Vlad Artenie[b]
[a] Department of Organic and Macromolecular Chemistry, Polytechnic Institute of Jassy, Jassy, Romania [b] "A. I. Cuza" University, Jassy, Romania

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Bioactive Polymers. XXVI. Immobilization of Pepsin on BIOZAN R

CRISTOFOR SIMIONESCU, SEVERIAN DUMITRIU, MARCEL POPA, and CRISTINA COMAN

Department of Organic and Macromolecular Chemistry
Polytechnic Institute of Jassy
6600 Jassy, Romania

VLAD ARTENIE

"A. I. Cuza" University
6600 Jassy, Romania

ABSTRACT

Pepsin was immobilized on BIOZAN R (Hercules) with dicyclohexylcarbodiimide as activator. The reaction product obtained has a protein content of 35-110 mg/g of polymer and a proteolytic activity between 20.85-28.75 μmol tyrosine/(L·min·g of polymer). The coupling reaction rate is maximum under the following conditions: pepsin/BIOZAN R ratio = 0.52 g/g, DCCI/BIOZAN R ratio = 0.2 g/g, pH = 3.4, reaction time = 4 h.

INTRODUCTION

The immobilization of proteases on synthetic polymers [1-10] renders biocatalytical systems suitable for applications in continuous enzymatic technologies. However, these products cannot be used therapeutically since most of synthetic supports are not biodegradable and consequently might cause intolerance.

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The natural polymers, and especially BIOZAN R (Hercules), can overcome these shortcomings and are consequently frequently used to obtain retarding and specific enzymatic drugs [11-14].

The coupling of pepsin to BIOZAN R, i.e., a polysaccharide of microbial biosynthesis [15], using dicyclohexylcarbodiimide (DCCI) as an activator, is reported in the present paper.

EXPERIMENTAL

The required amount of pepsin was dissolved under stirring in 5 mL buffer of the required pH according to the experimental protocol (Table 2). A solution of 0.08 g BIOZAN R in a 5-mL buffer solution of the same pH as that of the pepsin solution was separately prepared. The enzyme solution was added to BIOZAN solution, and the mixture was vigorously stirred. Finally, DCCI dissolved in 1 mL tetrahydrofuran (THF) was added. The coupling reaction was carried out for different times and at temperatures between 5 and 10°C. When the reaction was over, 20 mL acetone was added under stirring to remove the uncoupled pepsin, excess DCCI, and any dicyclohexylurea formed. The precipitate was washed with a buffer solution (0.2 M KCl and 0.2 M HCl, pH 2) until no protein was detected by the Lowry method [18] in the supernatant separated by centrifugation. Finally, the polymer was suspended in a buffer solution of pH 5.

The activity of the coupled enzyme was determined by the modified Anson method [17] consisting of the spectrophotometrical determination of the hydrolysis products (phenylalanine and tyrosine) released from hemoglobin under the action of pepsin.

RESULTS AND DISCUSSION

Previous investigations revealed that the activity of the coupling product and the amount of the covalently bound enzyme depend on several parameters, four of them being chosen for the present study of pepsin coupling, i.e., pepsin/BIOZAN R (g/g) ratio, DCCI/BIOZAN R ratio, pH, and reaction time.

A function of the following type was proposed to correlate the activity of the synthesized samples with the above parameters:

$$Y = a_o + \sum a_i x_i + \sum a_{ij} x_i x_j, \qquad i \leq j,$$

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

TABLE 1. Codes for the Independent Variables

Actual variable	Coded variable				
	-2	-1	0	1	2
Pepsine/BIOZAN R ratio, g/g, x_1	0.025	0.150	0.275	0.400	0.525
pH, x_2	1	2.5	4	5.5	7
DCCI/BIOZAN R ratio, g/g, x_3	0.2	0.45	0.7	0.95	1.2
Reaction time, h, x_4	4	11	18	25	32

A composed, rotatable, centrate, second-order experimental design was applied for finding the a_i and a_{ij} coefficients [19]. The values and codes of the variables are listed in Table 1. The experimental and calculated results are given in Table 2.

The experimental data in Table 2 were processed by the multiple regression method. The insignificant coefficients of the regression equation (a_{22}, a_{24}, and a_{33}) were eliminated by use of the "t" test [19]. The following equation describes the dependence of activity of the coupled enzyme on the reaction parameters:

$$A\ (\%) = 3.3 + 3.32x_1 - 2.26x_2 - 1.71x_3 - 0.64x_4 + 1.06x_1^2 - 2.9x_1x_2 - 1.56x_1x_3 + 0.67x_2x_3 - 0.51x_1x_4 + 1.6x_3x_4 + 0.65x_4^2.$$

The large values of the multiple correlation coefficient (0.878) and of the F factor (6.16) lead to the conclusion that the obtained equation describes the dependence of the enzymatic activity on the parameters under consideration fairly well.

By fixing three variables (in the center of the experimental domain, $x_i = 0$), information can be obtained regarding the influence of the fourth variable on the activity of the immobilized pepsin product.

The variation of the activity of the enzymatic product with the pepsin/BIOZAN R (g/g) ratio is depicted in Fig. 1 (Curve 1). The activity of the enzymatic product is seen to increase with increasing enzyme amount in the reaction mixture without attaining a maximum within the experimental domain.

TABLE 2. Experimental Design and Results

Variable				Activity μmol/(L·min·g)	
x_1	x_2	x_3	x_4	Experimental	Calculated
-1	-1	-1	-1	2.787	3.594
1	-1	-1	-1	28.717	20.194
-1	1	-1	-1	4.353	3.544
1	1	-1	-1	5.047	8.527
-1	-1	1	-1	2.504	1.248
1	-1	1	-1	7.553	9.102
-1	1	1	-1	1.370	1.383
1	1	1	-1	0.124	0.117
-1	-1	-1	1	1.890	0.129
1	-1	-1	1	14.596	14.688
-1	1	-1	1	1.725	0.796
1	1	-1	1	3.730	3.020
-1	-1	1	1	3.809	1.680
1	-1	1	1	12.192	9.689
-1	1	1	1	2.490	4.312
1	1	1	1	1.908	1.005
-2	0	0	0	0.460	0.889
2	0	0	0	11.502	14.182
0	-2	0	0	6.626	7.820
0	2	0	0	0.932	1.214
0	0	-2	0	4.898	6.732
0	0	2	0	0.888	1.273
0	0	0	-2	5.112	7.178
0	0	0	2	3.559	4.601
0	0	0	0	3.402	3.302
0	0	0	0	3.385	3.302
0	0	0	0	3.192	3.302
0	0	0	0	3.365	3.302
0	0	0	0	3.260	3.302
0	0	0	0	3.248	3.302
0	0	0	0	3.271	3.302

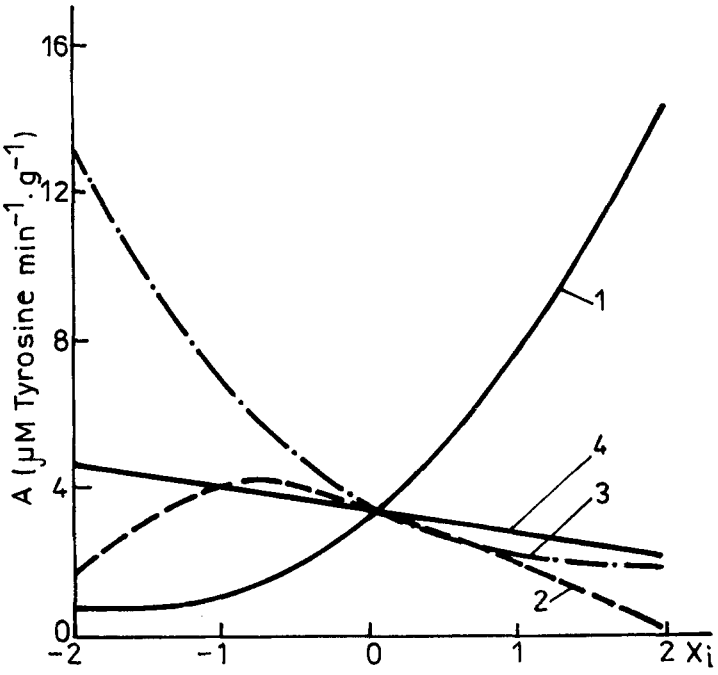

FIG. 1. Influence of reaction parameters on coupling of pepsin to BIOZAN R. (1) Pepsin/BIOZAN R ratio (pH = 4, DCCI/BIOZAN R ratio = 0.7, time = 18 h). (2) pH (pepsin/BIOZAN R ratio = 0.275, DCCI/BIOZAN R ratio = 0.7, time = 18 h). (3) DCCI/BIOZAN R ratio (pepsin/BIOZAN R ratio = 0.275, pH = 4, time = 18 h). (4) Time (pepsin/BIOZAN R ratio = 0.275, DCCI/BIOZAN R ratio = 0.7, pH = 4).

The reaction is influenced significantly by the pH value (Curve 2). The maximum coupling efficiency, expressed by the highest activity, lies at pH 3, regardless of the values of the other three variable pairs. At higher pH, a strong denaturation of both free and coupled enzymes takes place [20].

The DCCI/BIOZAN R (g/g) ratio is a determining parameter of the process (Curve 3). The data show the activity of the immobilized enzyme to decrease with increasing DCCI amount. Increasing the amount of DCCI promotes a side reaction of intra- and intermolecular condensation of the free and bound pepsin, which leads to irreversible denaturation of the enzyme. At a DCCI/BIOZAN R ratio of 0.2, all free carboxyl groups of the support are saturated through activation by DCCI, and the catalytical effect of pepsin intramolecular condensation is thus limited [21].

The activity of the reaction product is significantly influenced by the duration of the immobilization process. By prolonging the reaction

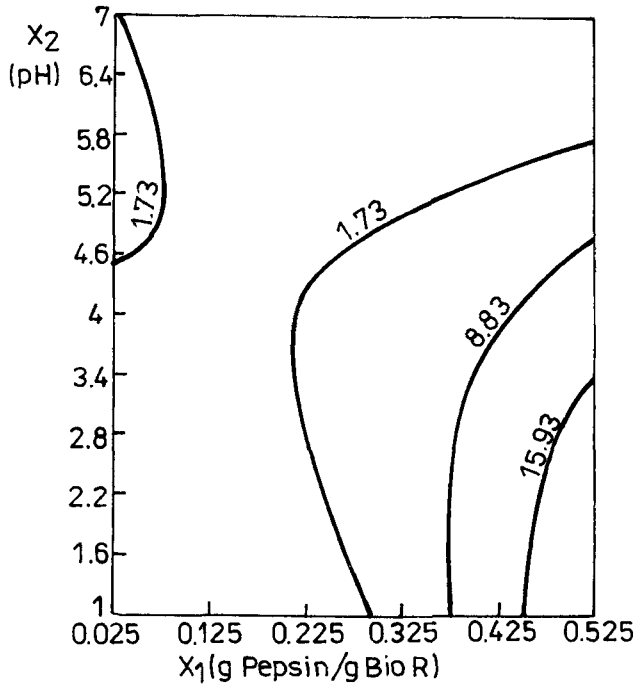

FIG. 2. Activity contours in the x_1-x_2 experimental plane (x_3 = 0.7, x_4 = 18).

time beyond 4 h (Curve 4), denaturation of the coupled enzyme occurs and, consequently, the protein fixed on the polymer cannot compensate for deactivation by the reagents in the system (DCCI, THF).

By correlating the reaction parameters with the activity of the immobilized enzyme, additional information can be obtained on better coupling conditions.

Thus, the constant-level curves in the x_1-x_2 experimental plane indicate rather high activities for high pepsin/BIOZAN R ratio values and low pH values (Fig. 2). Although an optimum is not obtained within the experimental domain, the most favorable conditions for maximum activities are obtained with higher x_1 and lower x_2 values.

Within the x_1-x_3 experimental plane, the efficiency of the pepsin coupling to BIOZAN R expressed by the hydrolytic activity of the enzymatic product is maximum with higher pepsin/BIOZAN R and lower DCCI/BIOZAN R ratios (Fig. 3).

A coupling maximum may also be achieved by working over the whole reaction time range with higher pepsin/BIOZAN R ratios (Fig. 4).

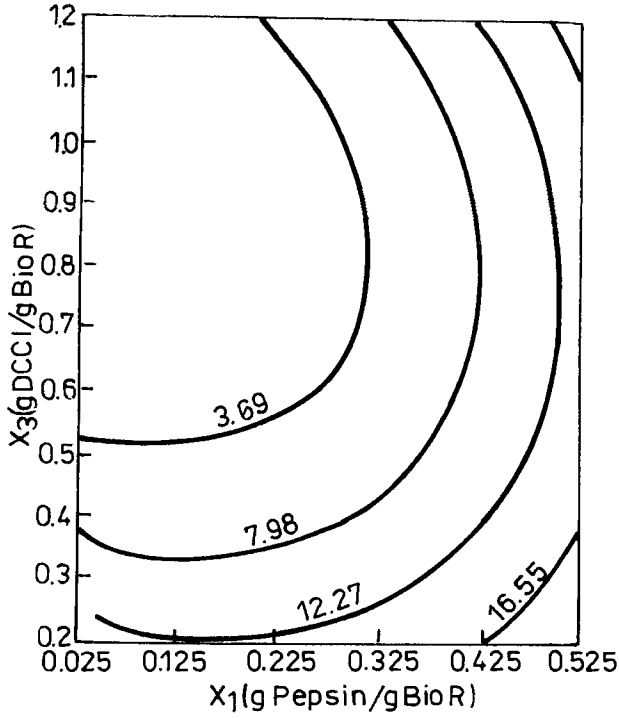

FIG. 3. Activity contours in the x_1-x_3 experimental plane (x_2 = 4, x_4 = 18).

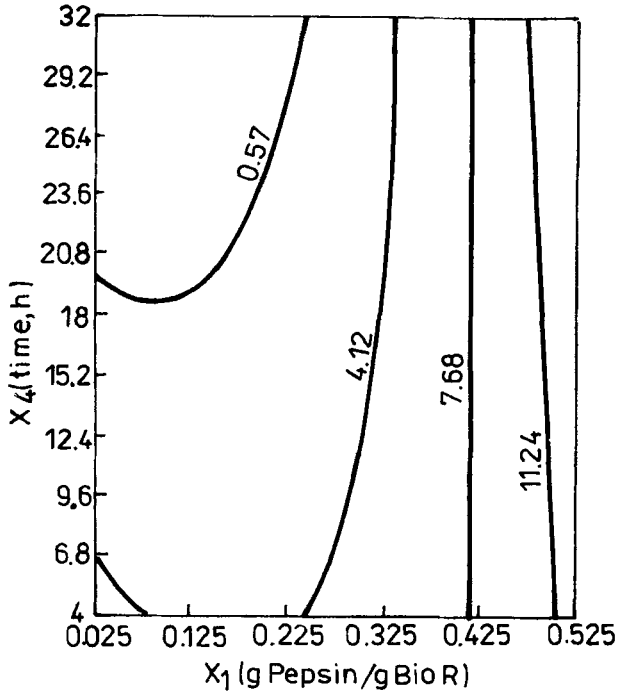

FIG. 4. Activity contours in the x_1-x_4 experimental plane (x_2 = 4, x_3 = 0.7).

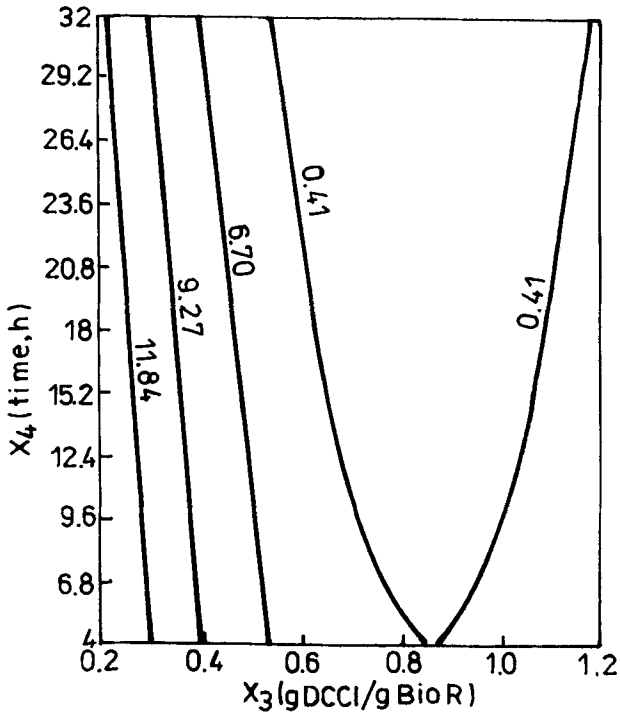

FIG. 5. Activity contours in the x_3-x_4 experimental plane (x_1 = 0.275, x_2 = 4).

From the x_3-x_4 experimental plane (Fig. 5), the conclusion may be drawn that the immobilized enzyme shows maximum activity at a minimum DCCI/BIOZAN R ratio value and a minimum reaction time within the experimental domain.

The immobilized pepsin products have enzyme contents between 35 and 110 mg enzyme/g dry substance and a proteolytic activity between 20.85 and 28.72 μmol tyrosine/(L·min·g). The coupling yield ranged between 4 and 11%.

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