Biopolymers

Bioactive Polymers.
28. Immobilization of Invertase on Carboxymethyl Cellulose Acid Chloride

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SUMMARY

The immobilization of invertase on carboxymethyl cellulose acid chloride and the factors influencing the reaction (enzyme/support ratio, duration and pH) are studied. The activity of both coupled and free enzymes varies with temperature and pH of the incubation reaction. The values of the Michaelis–Menten constant are higher for the immobilized enzyme.

INTRODUCTION

The immobilization of enzymes is a rather new investigation field intensively studied at present. The applied techniques belong to three principal categories: reticulation, encapsulation, coupling on different supports (by physical absorption, ionic and covalent bonding)(WEETALL and SUZUKI, 1975; MOSBACH, 1976; CHIELLINI and GIUSTI, 1983).
The investigations on invertase, an enzyme belonging to hydrolases, resulted in its immobilization by the above mentioned methods (SIMIONESCU et al., 1984; LOPEZ-SANTIN et al., 1983; MON-SAN and DURAND, 1971; IMAI et al., 1983; USAMI and KURATSU, 1973 FUKUI and CHIBATA, 1981).
In the present paper the immobilization of this enzyme by covalent bonding on the acid chloride of carboxymethyl cellulose is reported. This support is advantageous for its high reactivity at low temperatures which prevents the denaturation of the enzyme during the coupling.

EXPERIMENTAL

The immobilization of invertase is based on the condensation of the enzyme amino groups with the chloroanhydride ones on the support polymer:
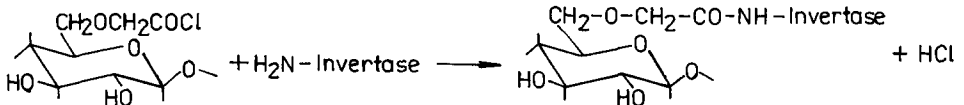
To assure the retention of the released HCl basic pH were used, this being a reaction parameter. Carboxymethyl cellulose (CMC-Cl) was prepared according to literature method (SIMIONESCU et al., 1982), showing a substitution degree with chloroanhydride groups $\gamma=0.37$ and a molecular weight $\bar{M} = 64500$, as measured viscosimetrically.
The necessary invertase amount was disolved under stirring in

20 ml phosphate buffer (0.02 M) of different pH values and then 0.1 g CMC-Cl were added. The mixture was maintained under stirring at $10\pm1^\circ$C for the required time. The reaction product was separated by centrifugation, washed with 200 ml buffer (pH=4.5) and distilled water and finally dried by lyophilizing.

The activity of the enzymatic samples was analysed photocolorimetrically (FEK 56 M, USSR spectrophotometer) by using 3, 5 dinitrosalicilic acid for the reaction stopping (IORDACHESCU and DUMITRU, 1980). The activity was calculated by means of the relation:

$$A = \frac{\mu\text{moles inverted sugar, sample} - \mu\text{moles inverted sugar, st.}}{15 \times \text{substance weight (g)}}$$

where 15 - incubation time (min).

The Michaelis-Menten constant was determined similarly by using saccharose solutions of different concentrations as substratum. The uncoupled enzyme as well as the enzymatic sample of the maximum activity were incubated at different temperatures and pH-values in order to follow the influence of these factors on the activity.

In order to corelate the activity of the enzymatic samples with the parameters of the coupling reaction the following function was proposed:

$$Y = a_o + \Sigma a_i x_i + \Sigma a_{ij} x_i x_j \quad , i \leqslant j$$

where:

Y - the enzyme activity; x_i - variables denoting the parameters under consideration; a_i, a_{ij} - regression coefficients. The regression coefficients were settled by programming the experiments according to the second order, composite, centrate, rotatable experimental design (COCHRAN and COX, 1968).

The experimental design, the obtained results and the codification of the parameters are listed in table 1. The experimental data were processed by the multiple regression method on a FELIX C - 256 computer.

RESULTS AND DISCUSSION

The following equation describes the dependence of the activity of the coupled enzyme on the reaction parameters:

$$A = 29.17 + 4.43\, x_1 + 3.53\, x_2 - 0.53\, x_3 - 0.62\, x_1^2 +$$
$$+ 0.94\, x_1 x_2 - 0.9\, x_2^2 - 0.15\, x_1 x_3 - 0.007\, x_2 x_3 - 1.87\, x_3^2$$

The high values of the multiple correlation coefficient (0.89) and of the F factor (4.226) lead to the conclusion that the equation describes well the dependence between the enzymatic activity and the coupling parameters. This dependence is illustrated in fig.1 for each variable by maintaining the other two constant (at the experimental domain center). As expected, the activity is noticed to increase with increasing enzyme amount in the reaction mixture (curve 2) without attaining a maximum within the experimental domain. The reaction duration also influences favourably the amount of the immobilized enzyme as expressed by the increase of the activity of the obtained products (curve 1). The reaction pH is also an important parameter determining a maximum activity at the value of 7.5 (curve 3). It is supposed that at pH values below 7.5 the HCl released by condensation is not entirely consumed, which causes the enzyme partial denaturation. At higher pH values the pronounced basic cha-

racter of the reaction medium, might lead also to the decrease of enzyme activity by denaturation. At the same time, the rate of the side reaction of chloroanhydride group hydrolysis increases significantly with increasing pH, thus diminishing the possibilities of enzyme coupling on the support.

Table 1. Experimental design and the obtained results.

| Nr.	The variable						The activity (μmoles/min.g)	
	Inv/CMC-Cl,g/g		Duration, h		pH			
	x_1	real	x_2	real	x_3	real	experiment.	calculat.
1.	-1	0.485	-1	1.21	-1	7.2	17.78	15.08
2.	1	1.614	-1	1.21	-1	7.2	31.66	33.84
3.	-1	0.485	1	3.29	-1	7.2	18.51	20.06
4.	1	1.614	1	3.29	-1	7.2	40.33	42.36
5.	-1	0.485	-1	1.21	1	7.8	14.16	14.21
6.	1	1.614	-1	1.21	1	7.8	26.04	24.97
7.	-1	0.485	1	3.29	1	7.8	14.22	16.52
8.	1	1.614	1	3.29	1	7.8	35.25	39.44
9.	-1.682	0.1	0	2.25	0	7.5	17.93	16.30
10.	1.682	2.0	0	2.25	0	7.5	39.40	39.09
11.	0	1.05	-1.682	0.5	0	7.5	16.66	19.17
12.	0	1.05	1.682	4.0	0	7.5	55.15	53.71
13.	0	1.05	0	2.25	-1.682	7.0	19.64	21.28
14.	0	1.05	0	2.25	1.682	8.0	25.83	23.83
15.	0	1.05	0	2.25	0	7.5	28.8	29.17
16.	0	1.05	0	2.25	0	7.5	29.3	29.17
17.	0	1.05	0	2.25	0	7.5	29.15	29.17
18.	0	1.05	0	2.25	0	7.5	29.17	29.17
19.	0	1.05	0	2.25	0	7.5	28.93	29.17
20.	0	1.05	0	2.25	0	7.5	28.85	29.17
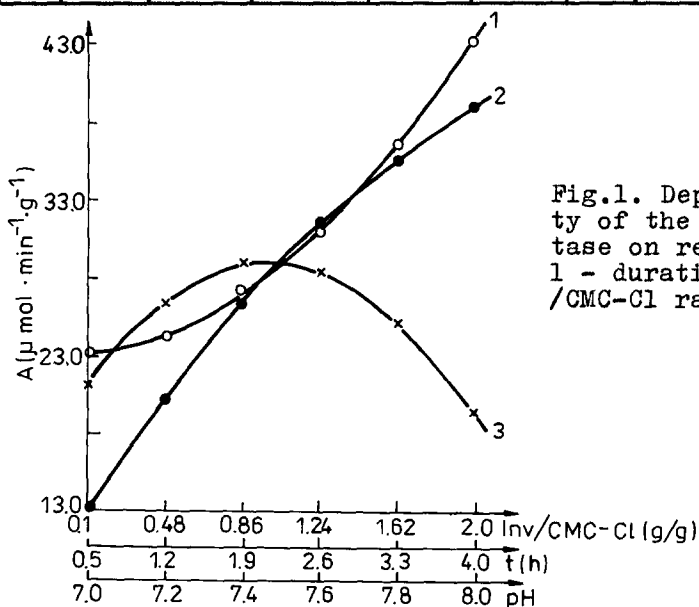
Fig.1. Dependence of activity of the immobilized invertase on reaction parameters. 1 - duration (h); 2 - Enzyme/ /CMC-Cl ratio; 3 - pH.

Important information can be obtained by the analysis of the
constant level surfaces expressing the correlated influence of
reaction parameters. Thus, fig.2 indicates closed activity va-
lues for low enzyme/support ratios and long durations or inver-
sely. The most favourable conditions for a maximum enzymatic ac-
tivitiy are given by the higher values of the parameters under
consideration although an optimum for the experimental domain is
not obtained. The highest activities of the enzymatic samples
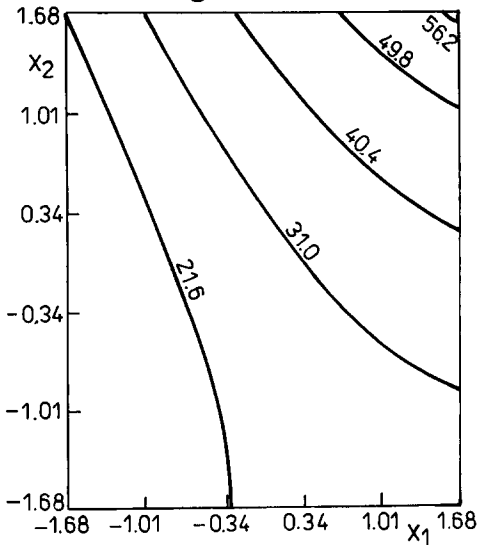are obtained for long reaction durations and pH = 7.5, as can
be seen in fig.3.

Fig.2. Constant level curves in
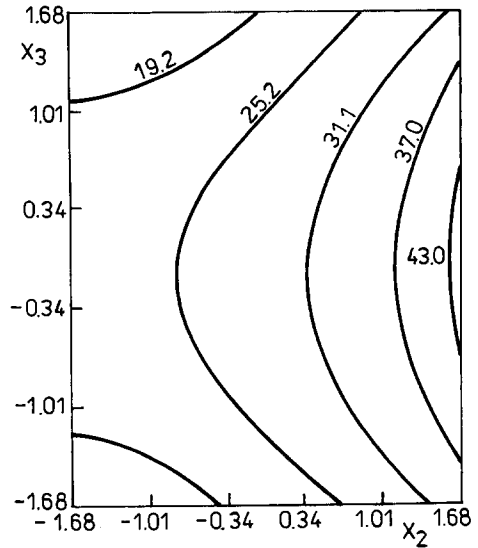the x_1 - x_2 experimental field.
(x_3 = 0).

Fig.3. Constant level curves
in the x_2 -x_3 experimental
field ($\bar{x}_1$ = 0).

The enzymatic activity is also influenced by the incubation con-
ditions. This fact was verified by determining the activity of
an enzymatic sample obtained under the most favourable immobi-
lization conditions, at different pH values (fig.4) in compa-
rison to the free enzyme.The activity is noticed to attain the
optimum value at pH = 4.5 in agreement with the literature va-
lues given for the free enzyme (PALMER, 1981).
By analysing the activity of the same sample for different in-
cubation temperatures the results presented in fig. 5 were ob-
tained. The immobilized enzyme shows the maximum activity at
40°C while for the free enzyme the temperature of 37°C is men-
tioned in literature. One has to mention that the free enzyme
shows a higher thermostability at temperatures above 40°C.
Information on the kinetics of the process catalysed by the free
and immobilized invertase were obtained from Linewaver - Burk
plot (fig.6). The Michaelis - Menten constant is higher for the
immobilized enzyme (K_M = 0.14 M) than for the free one (K_M =
= 0.05 M).
This fact is probably due to substratum concentration gradient
from the solution to the enzymatic sample particle caused by
the less intense stirring in the particle closed vicinity. Con-
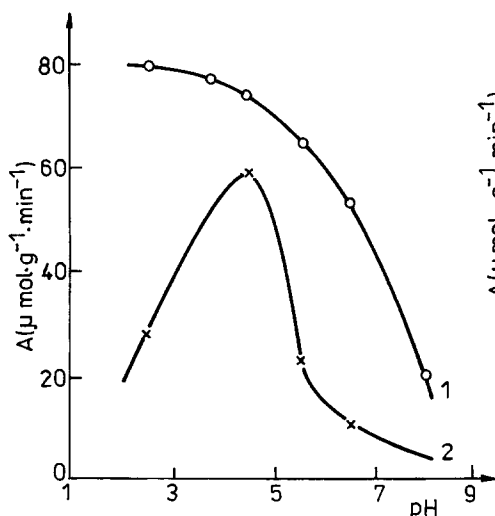sequently, the substratum diffusion is slower in the enzyme vi-
cinity.

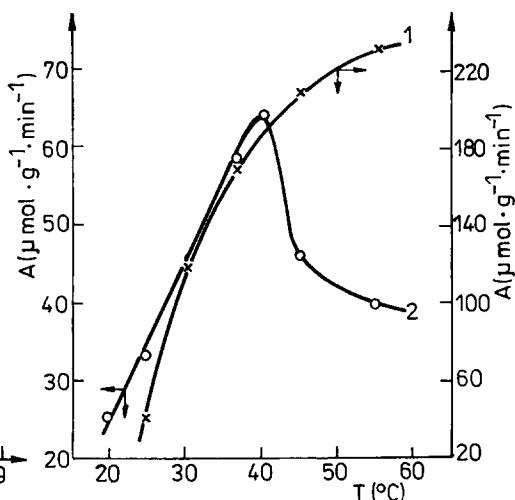Fig.4. Variation of the activity of free (1) and coupled (2) enzyme with the pH value of incubation reaction ($T = 37^\circ C$).

Fig.5. Variation of the activity of free (1) and coupled (2) enzyme with incubation temperature (pH = 4.5)

In order to have in the vicinity of the immobilized enzyme a substratum concentration equal to that at which the hydrolysis rate corresponding to the free enzyme is maximum, the solution of the substratum of the enzymatic sample had to be more concentrated. Consequently, the Michaelis constant increases for the coupled enzyme although it might be closed to the value characteristic of the free enzyme if the stirring is more intensive and the particle size reduced.
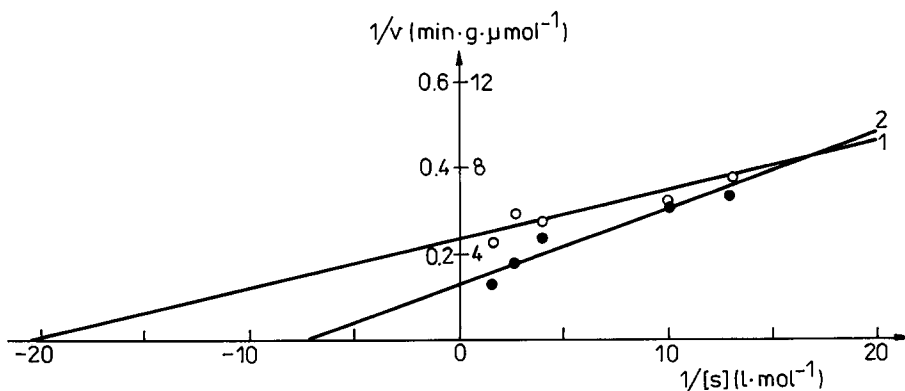

Fig.6. Linewaver - Burk plot for the free (1) and immobilized (2) invertase.

The diffusion of substratum to enzyme is also influenced by the electric charge of the support which may present remanent negative charge due to the dissociated carboxylic groups unconverted into chloroanhydride ones. The similar electric charges of

the support and substratum create an additional barrier which negatively influences the diffusion. It follows that the saturation of the immobilized enzyme would appear at a higher substratum concentration which explains the higher Michaelis constant in comparison to the free enzyme.

CONCLUSIONS

1. Invertase may be immobilized by covalent bonding on the acid chloride of carboxymethyl cellulose.
2. The immobilization reaction of invertase on the acid chloride of carboxymethyl cellulose is influenced by enzyme/support ratio, duration and pH of the reaction medium.
3. The activity of the immobilized enzyme depends on the incubation conditions, namely temperature and pH.
4. The Michaelis - Menten constant is higher for the immobilized enzyme.

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