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IMMOBILIZATION OF ALPHA - AMYLASE INTO GELATIN FILMS WITH VARIOUS CROSS - LINKERS

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Abstract This article reports the cross linking of α-amylase enzyme to gelatin by using formaldehyde, chromium (III) acetate, chromium (III) sulfate and potassium chromium (III) sulfate as hardeners. The effect of concentrations of cross linkers, enzyme and gelatin on the activity of immobilized enzyme were investigated.

INTRODUCTION

Immobilized enzymes are widely used for various biochemical and biomedical processes and in analytical applications. One of the main advantages of immobilization of α-amylase on cellulose triacetate films is that it can be used in continuous flow reactors. Because of their importance in industrial applications, immobilized enzyme reactors have been studied by many investigators[1,2].

In food technology, support material is much more important than other applications of immobilization. Gelatin was chosen as support material in this work, because it is a water soluble protein resulting from the partial hydrolysis of collagen[3] and it is abundant in the animal kingdom.

Reactive groups present in gelatin are primarily hydroxyl, carboxyl, and amino functions.Inorganic as well as organic compounds can harden gelatin. Among the former, which react in general with the carboxyl groups of the gelatin through their divalent metallic ions, the most often encountered are alums and chromium salts. In this study, chromium sulfate (CS) $[Cr_4(SO_4)_5(OH)_2]$; potassium chromium sulfate (PCS) $[KCr(SO_4)_2.12H_2O]$; and chromium acetate (CA) $[Cr(CH_3COOH)_3]$ are used as hardeners and they crosslink by connecting the ionized carboxyl groups.

Among numerous organic hardeners, the most frequently employed are the aldehydes, especially formaldehyde and glutaraldehyde. Hardening with aldehydes occurs by the formation of crosslinks between the amino groups of gelatin. In this study formaldehyde (FA) is used as hardener.

α-Amylase in the past has been reported to be entrapped by acrylamide /N,N-methylene-bisacrylamide copolymer[4], amicon PM 10 membrane[5] and adsorbed on collagen[6] and starch[7]; however, most of the former studies with this enzyme have dealt with its attachment to solid supports by covalent bonds such as carboxymethylcellulose[8], collagen[9] and cellulose[10]. Gelatin was used as support material in our previous work[11,12].

RESULTS AND DISCUSSION

To observe the effect of enzyme concentration on the activity of free and immobilized α-amylase, several enzyme concentrations (1-5 units/ml) were assayed. The reactions were carried out at 1.0 % starch concentration, pH 6.9 and 25° C . The results were presented in Figure 1.

% Maximum activities were calculated according to following formula;

ra:[activity of complex/(total activity of free enzyme used for coupling - activity loss by enzyme leakage)]

ma:maximum value of ra in series of experiments

% Maximum activity: ra x 100 / ma

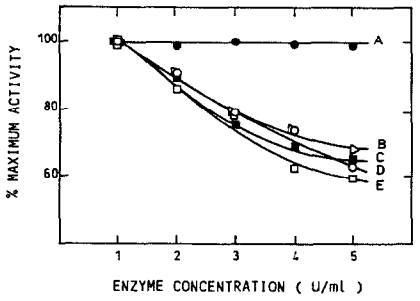

Figure 1. Effect of enzyme concentration on the activity of free and immobilized α-amylase. A:Free and B:Chromium sulfate, C:Chromium acetate, D:Potassium chromium sulfate, E:Formaldehyde

As seen in Figure 1.A , the activity of the free enzyme does not change considerably at different concentrations, in the range of 1-5 units/ml; however, maximum activities of the immobilized α-amylase film strips decreased with increasing enzyme concentration. As the enzyme concentration increased, leakage from film strips increased. Figure 1. B,C,D,E gives the maximum activities of the immobilized enzyme film strips prepared with α-amylase solutions of 1-5 units/ml gel.

Since gelatin and cross linker concentrations were kept constant in these experiments, this behavior can be explained by the possibility of increased enzyme-enzyme cross linking. The other proposed explanation of the results is that, with increased loading, the enzyme becomes oversaturated within the matrix pore, and thus restricts the product and the substrate diffusion[11,12].

It is believed that the extent of swelling of the gels would indicate how fast the substrate would reach the enzyme with conversion to product. Accordingly, α-amylase activity is expected to increase with increased swelling and the highest activity would be expected from the least concentrated solution[13] (the cross linker to gelatin concentration).

However in selecting the most suitable concentration of cross linking agents to be used in the rest of the work, highest activity was not the only criteria. The gel that produced the maximum enzyme activity and that leaked the enzyme least would be selected. The result of the swelling tests showed that the network structure must be quite loose for the high degree of swelling. On the other hand, loose network structure meant high levels of leakage[14] .

In order to observe the effect of cross linker concentration on gelatin insolubilized α-amylase activity, hardener concentrations were varied from 0.5 - 2.5 x 10^{-6} mol/ml gel. Enzyme and gelatin concentrations in the immobilization gel mixtures were 7.5 x 10^{-2} g/ml gel and 0.3 units/ml gel respectively. To find the amount of unbounded enzyme, leakage tests were performed. Immobilized enzymes were washed by phosphate buffer (pH 6.9) for 10 minutes, and the enzymatic activity was determined in the washing solutions. The results of the leakage tests and the final absorbances retained were presented in Tables 1-4 for different cross linkers.

The results showed that as the cross linker concentration in the immobilization gel mixture increased, the leakage of enzyme decreased. At the end of 90 minutes washing, the film strips insolubilized with CA and CS showed less activity at higher concentrations than strips prepared with PCS and FA. However the activities obtained for high cross linker concentrations were very low compared to the activity of the free enzyme. The absorbance of the native enzyme at 0.3 U/ml gel concentration was obtained as 1.500 (546 nm). The effect of cross linker concentration on the activity of gelatin insolubilized α-amylase can be seen in Figure 2.

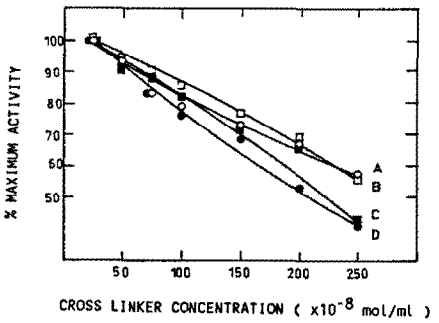

Figure 2. Effect of cross linker concentration on the activity of immobilized enzyme. A:Potassium chromium sulfate, B:Formaldehyde, C:Chromium acetate, D:Chromium sulfate

TABLE 1. Effect of chromium acetate concentration on leakage. Absorbance value of strips; A: after 9th washing; B: unwashed strips.

CROSS LINKERS (10^{-6} mol/ml gel)	Absorbance (546 nm)						
	Time of Washings (min)						
	10	30	50	70	90	A	B
0.25	0.085	0.075	0.069	0.065	0.050	0.165	0.272
0.50	0.079	0.070	0.065	0.054	0.041	0.150	0.242
0.75	0.072	0.058	0.059	0.048	0.039	0.145	0.228
1.00	0.063	0.061	0.056	0.044	0.021	0.135	0.214
1.50	0.048	0.043	0.038	0.023	0.012	0.118	0.192
2.00	0.032	0.037	0.020	0.008	0.000	0.108	0.174
2.50	0.018	0.021	0.015	0.004	0.000	0.070	0.116

TABLE 2. Effect of chromium sulfate concentration on leakage. Absorbance value of strips; A: after 9th washing; B: unwashed strips.

CROSS LINKERS (10^{-6} mol/ml gel)	Absorbance (546 nm)						
	Time of Washings (min)						
	10	30	50	70	90	A	B
0.25	0.081	0.075	0.042	0.029	0.025	0.162	0.225
0.50	0.065	0.044	0.039	0.027	0.017	0.149	0.187
0.75	0.044	0.033	0.026	0.020	0.010	0.135	0.169
1.00	0.037	0.030	0.024	0.017	0.007	0.123	0.145
1.50	0.028	0.025	0.018	0.010	0.005	0.111	0.132
2.00	0.017	0.012	0.010	0.005	0.000	0.102	0.112
2.50	0.015	0.011	0.009	0.003	0.000	0.066	0.084

TABLE 3. Effect of potassium chromium sulfate concentration on leakage. Absorbance value of strips; A: after 9th washing; B: unwashed strips.

CROSS LINKERS (10^{-6} mol/ml gel)	Absorbance (546 nm)						
	Time of Washings (min)						
	10	30	50	70	90	A	B
0.25	0.098	0.082	0.075	0.066	0.054	0.201	0.315
0.50	0.082	0.077	0.064	0.059	0.049	0.188	0.290
0.75	0.077	0.054	0.046	0.041	0.037	0.167	0.275
1.00	0.061	0.048	0.039	0.036	0.030	0.159	0.239
1.50	0.058	0.039	0.030	0.027	0.021	0.147	0.215
2.00	0.040	0.037	0.021	0.015	0.009	0.135	0.204
2.50	0.035	0.022	0.017	0.008	0.001	0.115	0.175

TABLE 4. Effect of formaldehyde concentration on leakage. Absorbance value of strips; A: after 9th washing; B: unwashed strips.

CROSS LINKERS (10^{-6} mol/ml gel)	Absorbance (546 nm)						
	Time of Washings (min)						
	10	30	50	70	90	A	B
0.25	0.112	0.087	0.065	0.048	0.043	0.187	0.307
0.50	0.101	0.078	0.060	0.039	0.038	0.174	0.285
0.75	0.095	0.059	0.039	0.034	0.033	0.169	0.257
1.00	0.080	0.052	0.045	0.039	0.031	0.160	0.231
1.50	0.061	0.048	0.034	0.031	0.026	0.143	0.212
2.00	0.056	0.040	0.031	0.025	0.022	0.129	0.195
2.50	0.043	0.028	0.020	0.017	0.010	0.104	0.158

Although less leakage was obtained with high hardener concentrations, increasing cross linker concentration decreased the activity of immobilized enzyme. This observation was attributed to two reasons. Excess of cross linkers inactivated some of the immobilized enzyme molecules by blocking the active site of the enzyme and the uneven distribution of water in hardened gelatin. Most of the water retained is found in the voids of its characteristic spongy structure[15]. As a result, the substrate and the reaction products, diffusing in and out of gelatin - enzyme system are slowed down. The effect of diffusion limitation decreases the activity of enzyme more drastically at higher cross linker concentrations. Minimum leakage with considerable activity was obtained at 2.0×10^{-6} mol/ml gel cross linker concentration. In these washing experiments, film strips prepared with CA and CS showed less leakage whereas higher enzymatic activity and enzyme leakage was observed for strips insolubilized with PCS and FA. Immobilizations with all of the cross linkers showed that leakage was minimal and the activity of the film strips were considerably high.

To determine the effect of gelatin concentration on activity and stability of immobilized α-amylase, the conditions other than gelatin concentration were kept constant. Gelatin concentrations of $2.5\text{-}10 \times 10^{-2}$ g/ml gel at 2.0×10^{-6} mol/ml cross linker concentration were investigated. Figure 3 shows the relationship between the concentration of photographic gelatin and maximum enzymatic activity, where immobilizations were realized at different concentrations of gelatin with a constant enzyme load (0.3 U/ml gel).

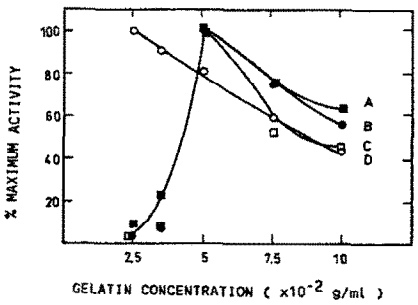

Figure 3. Maximum activities of immobilized α-amylase prepared by different gelatin concentrations. A:Chromium acetate, B:Chromium sulfate, C:Potassium chromium sulfate, D:Formaldehyde

At low gelatin concentrations (2.5, 3.5 and 5.0×10^{-2} g/ml gel) the film strips were mechanically weak and the gelatin spots were ruptured after 6 or 7 uses. At high gelatin concentrations, mechanically strong films with low enzymatic activity were obtained. As a result the most suitable gelatin concentration was found to be 7.5×10^{-2} g/ml gel.

In summary, the following conclusion can be drawn from the present work. Immobilization of α-amylase into photographic gelatin by chemical cross linking with formaldehyde, chromium acetate, chromium sulfate and potassium chromium sulfate were achieved for the first time, where the cellulose triacetate films were used as the supporting base.

EXPERIMENTAL

MATERIALS- α-Amylase EC.3.2.1.1 (1,4-α-D-glucan glucanohydrolase) lyophilized from porcine pancreas (30.000 U/vial or 300 U/mg lyophilisate) was purchased from E.Merck.

Soluble starch (Art 1253) was the product of E.Merck and used as the substrate.

Photographic gelatin was obtained in granular form from Croda.

The cellulose triacetate films were obtained from Du Pont De Nemours. The films with 102 μm thickness were supplied as precoated with photographic gelatin.

Chemicals used in the preparation of buffers and cross linking agent were supplied from Merck. Other chemicals were analytical grade products of different origin.

METHOD- The enzymatic activity was analyzed photocolorimetrically[16]. Before the start of the assay, 0,1 ml of enzyme sample (as free enzyme solution; 0,3 U) and 1 ml of phosphate buffer (20 mM, pH 6,9) were placed in a test tube. After equilibrating the temperature to 25° C, 1 ml of substrate solution (1 % , w/v), starch was added into the tubes and the assay was accomplished. Then, the tubes were incubated for 10 minutes at 25° C and the reaction was stopped by adding 2 ml of 3,5-dinitrosalicylic acid solution. The solutions were heated in boiling water bath for 5 min. cooled and the absorbances were read after 20-60 min. The samples were analyzed photocolorimetrically at 546 nm (LKB Nova Spect II spectrophotometer) against a blank solution, which was prepared in the same manner lacking the enzyme.

The enzymatic activity was calculated by using the equation[16] :

Volume Activity = 4950 x δE [U/L]

[U/min] = [U /L] x [L/min]

The photographic gelatin (0.750 g) used for the immobilization of α-amylase was swelled in 10 ml of phosphate buffer (20 mM, pH 6.9 at room temperature for 30 min.). The mixture was heated at 50° C until all the gelatin dissolved. The temperature was lowered to 32° C and 0.3 ml of enzyme solution (105 μg, 30 U) was added into the gelatin mixture. Then the required amount of FA, CA, CS or PCS (200 μl, 2 x 10^{-6} mol/ ml gel) was added. The mixture was stirred constantly at 32° C while 0.1 ml aliquots were taken and placed onto cellulose triacetate film strips (each spot containing 0.3 units of enzyme).

The film strips with enzyme gelatin spots were left to dry at room temperature for 24 hours, to allow the completion of cross linking. The diameter of the spots after drying was ca. 1.0 cm. The dimensions of the film strips were 20 x 1.2 cm with a thickness of 100 μm. From every enzyme - gelatin batch prepared, an average of 25 - 30 strips were made.

The film strips for blank were prepared as described above without the addition of the enzyme solution.

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