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POLYESTER FILM STRIPS COATED WITH PHOTOGRAPHIC GELATIN CONTAINING IMMOBILIZED INVERTASE

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ABSTRACT

Invertase was immobilized into photographic gelatin by chemical crosslinking with formaldehyde and chromium (III) acetate. Polyester film strips were coated with invertase-gelatin mixture which increased durability during consecutive immersions into reaction media. The effect of gelatin, crosslinker and enzyme concentrations on activity were studied. Enzyme leakages from immobilized invertase film strips were controlled by washing with EDTA solutions. The activities of washing solutions were measured spectrophotometrically to determine unbounded enzyme concentration. Effect of use number on activity was also determined. Percent activity of immobilized invertase was lower than that of free enzyme. The activity however retained even after 10 washings (170 minutes) followed by 9 use in 18 weeks.

INTRODUCTION

Immobilization of various enzymes, has been studied in detail during the past 30 years. The advantages of using immobilized enzymes in food, drug and analytical applications are well known [1-2]. Various physical and chemical immobilization techniques are summarized in reviews and books [3-6]. There are some disadvantages of immobilization techniques which need to be minimized prior to large scale applications. A decrease in enzymatic activity is observed in all entrapping methods, due to the hindrance caused on diffusion of substrate by the polymer matrix [7]. However immobilized enzymes can find applications in industry due to the advantages as reusability, continuous operational modes, rapid termination, product formation control and high efficiency in multistep reactions etc [2].

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Immobilized invertase can be used in food industry to prevent crystallization when it is used in hydrolysis of sucrose to yield sugar mixture. Hydrolyzed sugar mixture obtained by invertase will have an advantage of being colorless compared to the colored products obtained by acid hydrolysis [8].

Immobilization of invertase on porous glass [9], corn grits [8], styrene divinylbenzene copolymer [10], diazonium salt of 4-aminobenzoyl cellulose [11], poly (ethylenevinyl alcohol) membrane [12], polyacrylamide [13], and in photo crosslinkable poly (vinyl alcohol) [7] were reported.

Scardi et al. [14] recently reported that when invertase was immobilized in gelatin the loss of activity was due to internal diffusion limitations when formaldehyde was used as crosslinker.

In the present work, chromium acetate and formaldehyde were used as crosslinking agents to insolubilize photographic gelatin invertase system. Polyester film strips were used as supporting base for immobilized invertase in gelatin. Enzyme leakage, reuse number and effect of concentration were studied.

EXPERIMENTAL

Materials

Invertase was isolated from dry yeast (Pak Maya food Co.). 50 grams of dry yeast was dissolved in 150 mL of freshly prepared $NaHCO_3$ solution and allowed to autolyze for 24 h at 40 - 45 °C. The inactive proteins were precipitated by addition of picric acid into centrifuged solution. The solution containing the precipitate was centrifuged, and chilled acetone was added into supernatant to precipitate invertase enzyme below 0 °C. Invertase was dissolved in 0.1 % EDTA pH 7.2, and purified by dialysis at 0 °C according to procedure given by Hestrin et al [16]. Later this enzyme solution was diluted by 0.5×10^4, 1.0×10^4, 2.0×10^4 and 4.0×10^4 times in 0.1 % EDTA pH 7.2, and used in the further experiments.

Photographic gelatin was obtained in granular form from Croda and used without further purification.

Polyester films were obtained from Dupont. The films with 100 mm thickness were supplied as precoated with photographic gelatin. The precoated gelatin allowed strong adhesion of invertase-gelatin mixture on the film base.

Sucrose was product of Sigma and the other chemicals were obtained from Merck.

Methods

Photographic gelatin (0.75 g) was swelled in 10 mL of 0.1 % EDTA pH 7.2 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 0.7 mL of invertase at previously specified concentrations was added into gelatin solution. Then the required amount of chromium (III) acetate or formaldehyde solutions were added as crosslinking agent. The mixture was stirred constantly at 32 °C while 0.1 mL aliquots were taken and placed on polyester film strips.

The film strips with enzyme-gelatin spots were dried at room temperature for 24 hours, to allow the completion of crosslinking. The diameter of the spots after drying was ca. 0.9 cm. The dimensions of the film strips were 20 x 1.2 cm with thickness of 100 μm. From every enzyme-gelatin batch prepared, an average of 25-30 strips were made. An automatic pipette was used to obtain uniform 0.1 ml spots.

The activities of free and immobilized invertase were determined by Nelson method [17] using a Hitachi 200 D spectrophotometer.

Each film strip was immersed into separate test tubes containing 1.5 mL of EDTA pH 7.2 and 0.4 mL of acetate buffer pH 4.6 covering the spots completely. Following the preincubation of 2 min at 25 °C the substrate was added to each tube. The amount of sucrose (0.3 M) solution added, was 0.1 mL. The solutions were incubated for 15 min at 25 °C, after which the film strips were removed. The activity was determined from the amount of reducing sugar produced.

RESULTS AND DISCUSSION

Hardening of gelatin [18, 19] by formaldehyde and chromalum has been used in photographic industry for many years. The photographic emulsions prepared by hardened gelatin allows the diffusion of developing solutions into films with a controlled rate. The insolubility of hardened gelatin also increases the durability of photographic films and papers in developing solutions. The excellent properties of insolubilized gelatin in solution, made it an irreplacable support material in photographic industry even today.

Gelatin granules swollen in water and dissolved above 35 °C, form a colloidal solution, a sol which gelifies an cooling. In the absence of hardening agents, this process is entirely reversible, therefore it can not be used in immobilization of enzymes without hardeners. Introduction of hardening agents into gelatin solution not only insolubilizes the gel even at high temperatures but considerably reinforces the mechanical resistance of dry as well as wet gelatin layers. This is indispensible mainly for enzyme immobilization on film strips which requires a succession of alternative immersions into alkaline or acid solutions.

In the present work photographic gelatin was hardened by formaldehyde and chromium acetate to immobilize invertase. For insolubilization of gelatin, the following reaction can be written in presence of formaldehyde;

$$\mathbf{G}\text{—}\ NH_2 + HCHO + H_2N\text{—}\ \mathbf{E} \rightarrow \mathbf{G}\text{—}\ NH\text{-}CH_2\text{-}HN\text{—}\ \mathbf{E} + H_2O$$

TABLE 1. Enzyme leakage test for immobilized invertase film strips washed in 0.1 % pH 7.2 EDTA. Invertase was diluted 5 x 10^3 times prior to immobilization unless otherwise indicated.

Number of washing	ABSORBANCE [a]			
	[FA]=3x10^{-1} M	[FA]=3x10^{-1} M	[FA]=1.2x10^{-1} M	[Cr^{+3}]=2.5x10^{-2}M
1	0,003 [b]	-	0,008	0,020
3	-	-	0,008	0,021
5	-	-	-	0,018
7	-	-	-	0,015
9	-	-	-	0,010
10	-	-	-	-

[FA] = Formaldehyde concentration

a) An absorbance value of 0,744 was obtained for free enzyme diluted 10^4 times.

b) Enzyme diluted 10^4 times

When chromium acetate was used as hardener immobilization can take place according to following reaction;

$$\mathbf{G}\text{—}COOH + Cr(OCO\text{-}CH_3)_2(OCO\text{-}CH_3) + HOOC\text{—}\mathbf{E} \rightarrow Cr(OCO\text{—}\mathbf{G})(OCO\text{-}CH_3)_2 + \mathbf{E}\text{—}OCO\text{-}CH_3 + CH_3COOH$$

along with other possible combinations.

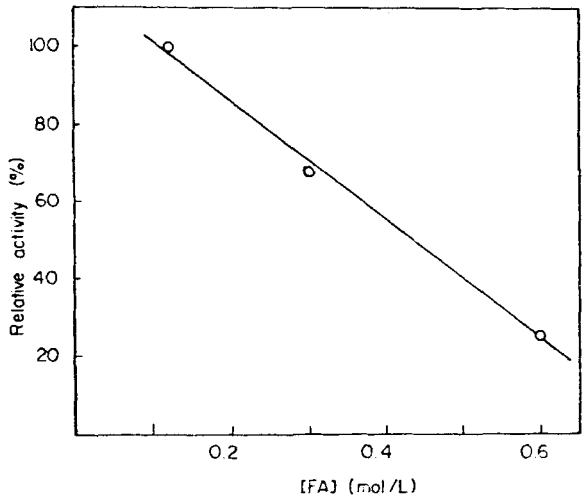

Figure 1. Effect of formaldehyde concentration [FA] on relative activity of immobilized invertase film strips.

Immobilized invertase film strips were tested for enzyme leakage by consecutive washings in 0.1 % EDTA solution pH 7.2. The washing solutions were analyzed for enzyme content by Nelson method. Each washing was carried out at 5°C for 17 minutes. As seen in Table 1 the absorbances measured spectrophotometrically are very small or negligible for the washing solutions. When the aborbances are converted to activity, the values are nearly zero for all washings. This observation shows that enzyme leakage is minimal or nil from the immobilized invertase film strips.

The effect of formaldehyde concentration on the activity of immobilized invertase is given in Figure 1.

As seen in the figure the activity decreases as the amount of formaldehyde increases. This observation can be due to two reasons. Excess of formaldehyde can inactivate some of the immobilized enzyme molecules by blocking the active site of enzyme during crosslinking. On the other hand, in hardened gelatin, water is not distributed homogeneously. Most of the water retained is found in the voids of its characteristic spongy structure [15]. As a result of this the substrate and the reaction products, diffusing in and out of gelatin-enzyme system, are slowed down. This hindrance is due to the presence of solid portions of the hardened gelatin. The effect on diffusion, decreases the activity of enzyme more drastically at high formaldehyde concentrations. Minimum leakage with highest activity was obtained at 1.2×10^{-1} mol/L formaldehyde concentration which was used for the rest of the work.

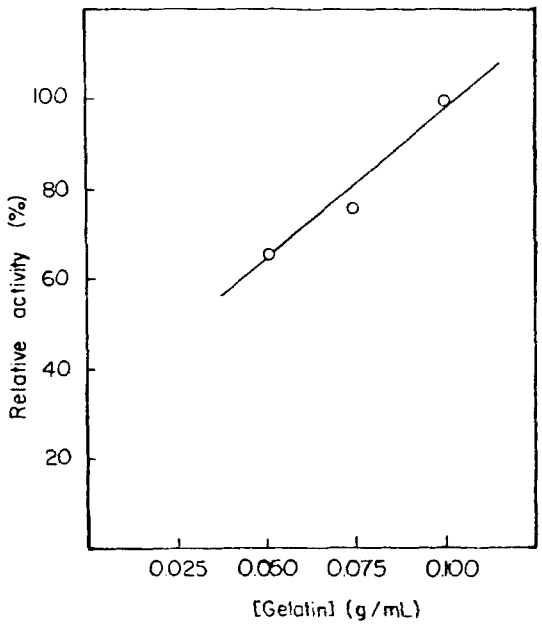

Figure 2. Effect of photographic gelatin concentration on relative activity of immobilized invertase film strips.

As seen in Figure 2 when gelatin concentration decreases, the activity of immobilized enzyme decreases too. Since the concentration of formaldehyde was kept constant at 1.2×10^{-1} mol/L, the ratio of formaldehyde/gelatin increased as gelatin concentration was lowered. As a result of this the activity decreases due to the reasons given for the case of high formaldehyde concentration.

Immobilization of invertase in photographic gelatin was also accomplished by using chromium (III) acetate as hardener. Relative activities of invertase versus enzyme concentration for formaldehyde and chromium (III) acetate hardened film strips and free enzyme are given in Figure 3.

The concentration of the purified enzyme stock solution was assumed to be 1 in Figure 3.

As seen in Figure 3 A activity of free enzyme does not change considerably upon dilution, in the range of 5×10^{3} to 40×10^{3} times of dilution. The relative activity of immobilized invertase however increases from 25 % up to 72 % by diluting the enzyme solution four times, in case of chromium (III) acetate hardening. Figure 3B gives, the relative activities of immobilized enzyme film strips prepared by

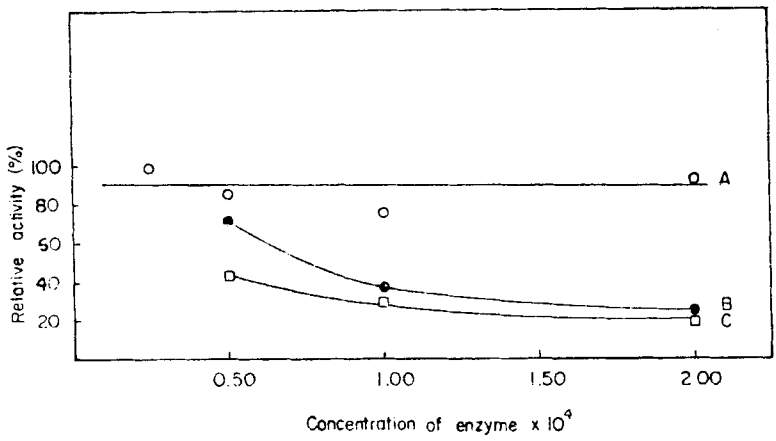

Figure 3. Relative activities of immobilized enzyme film strips prepared by invertase solutions at various dilutions. A: Free enzyme, B: Chromium (III) acetate (2.5×10^{-2} mol/L) hardening and C: Farmaldehyde (1.2×10^{-1} mol/L) hardening

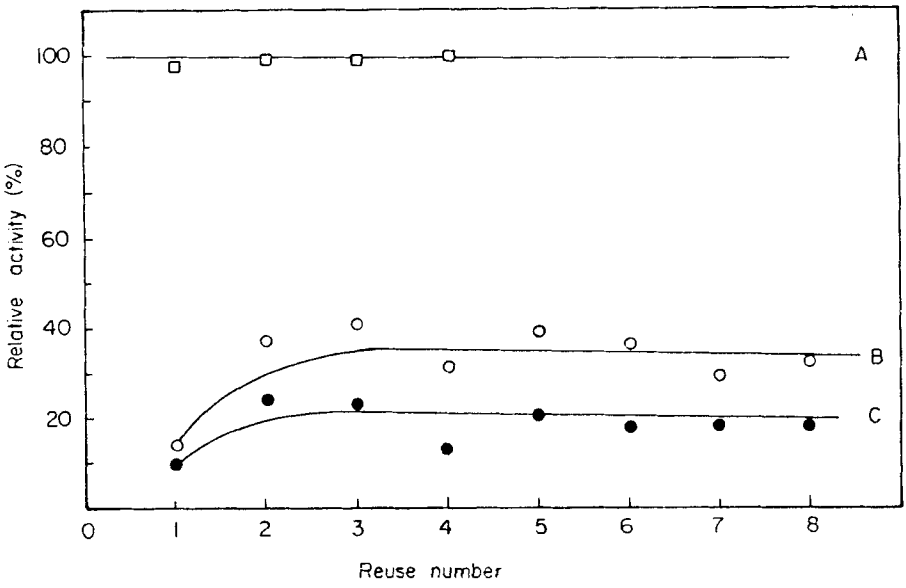

Figure 4. Relative activity versus reuse number plots for immobilized invertase film strips hardened by farmoldehyde (3.0×10^{-1} mol/L). A: 10000 times diluted free enzyme solution, film strips prepared from B: 10000 times and C: 5000 times diluted enzyme solutions.

invertase solutions diluted 20×10^3, 10×10^3 and 5×10^3 times, with activities of 72 %, 38 % and 25 % respectively. When formaldehyde was used as hardening agent, dilution of enzyme solution increased the relative activity of immobilized invertase also (Fig 3C). As seen in the figure, relative activity increases from 20 % to 44 % upon four times of dilution of enzyme solution. In Figure 3C the relative activities are given as 44%, 30% and 20% for immobilized invertase film strips prepared from 20×10^3, 10×10^3 and 5×10^3 times of diluted enzyme solutions respectively. This behaviour can be explained by a decrease in enzyme-gelatin crosslinking when concentrated enzyme solutions are used in immobilization. The gelatin and croslinker concentrations were kept constant and only the enzyme concentration was changed. Therefore in Figure 3B and 3C as the concentration of enzyme solution increased enzyme-enzyme crosslinking should increase, causing a decrease in relative activity.

Percent immobilization versus use number plot is given in Figure 4 for invertase immobilized by formaldehyde hardening. Figures 4A and 4B represent percent immobilization versus use number for film strips prepared by 10×10^3 and 5×10^3 times diluted invertase solutions respectively. As seen in the plots there is a slight increase in percent immobilization after first use which does not change considerably afterwords. The stability in percent activity even after 8^{th} use shows that invertase is immobilized mainly by covalent bonding rather than physical entrapment. Percent activity of immobilized invertase film strips prepared from 10×10^3 times diluted enzyme solution is higher than that of strips prepared from 5×10^3 times diluted enzyme solution. This behaviour is consistent with the results given in Figure 3. Repeated use of strips did not change this trend as seen in Figure 4. The strips were washed 10 times in EDTA initially, used once then let to stand for a week at room temperature and then used once more and so on. Therefore each point in Figure 4 represents measurement of activity of the same strip after one week of resting. Following the last use the strips were kept at room temperature for 10 more weeks and the percent activity was found to be unaffected (18 weeks totally).

In conclusion we have shown that invertase can be immobilized in photographic gelatin by formaldehyde and chromium (III) acetate. Immobilized invertase film strips are durable and can be reused many times without loss of activity. The enzyme coated strips can be stored in used or unused form for 18 weeks and retain their activity. The washing solutions did not have considerable enzyme activity which indicated that invertase was mainly immobilized by chemical crosslinking rather than physical entrapment.

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