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STABILIZATION EFFECT OF POLYVINYL ALCOHOL ON HORSERADISH PEROXIDASE, GLUCOSE OXIDASE, β-GALACTOSIDASE AND ALKALINE PHOSPHATASE

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SUMMARY

The stabilization effect of different molecular weights of polyvinyl alcohol (PVA) upon solutions of commonly used analytical enzymes was studied. It was found that various PVA conditions could stabilize the activity of horseradish peroxidase and glucose oxidase but not β-galactosidase or alkaline phosphatase. It is expected that PVA can stabilize conjugate forms of glucose oxidase as well as horseradish peroxidase (as previously shown) but not the conjugate forms of β-galactosidase or alkaline phosphatase.

INTRODUCTION

Enzyme immunoassays (EIA) are a major biotechnology product that depend upon the activity of enzymes conjugated to immunoreactants (antibodies or antigens) for the detection of antigens (e.g., pathogens, pollutants and toxins) or antibodies (e.g., for serodiagnosis) in samples. The instability of enzyme conjugates necessitates the use of refrigeration or refrigerant during storage and use, making EIA expensive and field EIA unfeasible. Previously, we have shown that polyvinyl alcohol (PVA) of molecular weight 9,000-10,000 and 124,000-186,000 at concentrations as low as 0.5 % (w/v) provided complete stabilization of horseradish peroxidase conjugates stored in solution at 30 °C (Boyd and Yamazaki, 1994). In addition to horseradish peroxidase, there are other enzymes such as glucose oxidase, bacterial β-galactosidase, and bovine alkaline phosphatase which are commonly used for EIA as conjugates in which the enzymes are conjugated to

immunoreactants through a number of different conjugation methods. Therefore, in the present paper we have studied the stabilization effect of different molecular weights of PVA upon horseradish peroxidase, glucose oxidase, β-galactosidase, and alkaline phosphatase.

MATERIALS AND METHODS

Materials

The following materials were obtained from Sigma Chemical Company: horseradish peroxidase (HRP) (P-6782), glucose oxidase (GO) (G-7141), bacterial β-galactosidase (β-gal) (G-6762), bovine alkaline phosphatase (AP) (P-0405), 3,3'5,5'-tetramethybenzidine (TMB) (T-2885), p-nitrophenol phosphate (p-npp) (Sigma No. 104), O-nitrophenyl-β-D-galactopyranoside (ONPG) (N-1127), polyvinyl alcohol (PVA) (MW 30,000-70,000; 87-89 % hydrolysis) (P-8136), and PVA (MW 70,000-100,000; 99+ % hydrolysis) (P-1763). Aldrich Chemical Company supplied the PVA (MW 9,000-10,000; 80 % hydrolysis) (36,062-7), PVA (MW 13,000-23,000; 87-89 % hydrolysis) (34,840-6), and the PVA (MW 124,000-186,000; 99+ % hydrolysis) (36,306-5). The different species of PVA were all of laboratory grade. The Micro-O-Protect (a mixture of bromonitrodeoxane and 2-methyl isothiazolone) was acquired from Boehringer Mannheim (1585720).

Effect of PVA on the Activity of HRP, GO, AP, and β-Gal

HRP (1 μg), GO (10 μg), β-gal (200 μg), and AP (1 μg) were suspended individually in 1 ml of PBS (0.01 M sodium phosphate pH 7.3 buffer in 0.85 % NaCl) with 0.1 % (v/v) Micro-O-Protect (PBSM), or 1 ml of various PBSM solutions each containing a different molecular weight of 5 % (w/v) PVA. The HRP, GO, and β-gal solutions were incubated at 30 °C, with the AP solutions being incubated at 40 °C. At regular time intervals, the different solutions were assayed for enzyme activity.

Assay of Enzyme Activity

(i) HRP:

After 0 to 10 days of incubation, HRP samples were removed from each condition and diluted 100 times in PBS. Fifty μl of the diluted HRP suspension was mixed with 1 ml of the TMB indicator system in a 16 mm x 100 mm test tube and shaken at 180 RPM (on a New Brunswick Gyrotary Shaker) for 10 min at 30 °C. The HRP reaction was stopped by adding 250 μl of 2 N H_2SO_4 and then the resultant colour was measured at 450 nm. The

TMB indicator system was prepared by mixing 2.5 ml of TMB (2 mg/ml of ethanol) with a solution consisting of 0.21 g citric acid·H_2O, 0.42 g EDTA·4 Na, 0.03 g $NaBO_4$·4 H_2O, and 100 ml distilled water (dH_2O).

(ii) GO:

After 0 to 4 days of incubation, GO samples were removed from each condition and diluted 40 times with PBS. One-hundred μl of the diluted GO suspension was mixed with 1 ml of the glucose oxidase assay solution in a 16 mm x 100 mm test tube and shaken at 180 RPM for 30 min at room temperature. The resultant colour was immediately measured at 510 nm. The glucose oxidase assay solution was prepared according to Blais and Yamazaki (1992) as follows: 0.6 g of dibasic and 0.1 g of monobasic anhydrous potassium phosphate, 10 g of D-glucose, 0.5 g of potassium iodide, 2.5 g of PVA (MW 30,000-70,000), and 60 mg of sodium molybdate dihydrate were dissolved in 100 ml dH_2O immediately prior to use.

(iii) β-Gal:

After 0 to 6 hours of incubation, β-gal samples were removed from each condition and diluted 50 times with β-gal enzyme diluent (0.01 M Tris-HCl (pH 7.5) containing 0.01 M $MgCl_2$, 0.01 M mercaptoethanol, and 0.01 M NaCl). One-hundred μl of the diluted β-gal suspension was mixed with 0.3 ml of 1.0 M mercaptoethanol, 1.1 ml of dH_2O, 1.0 ml of 3.0 M sodium phosphate buffer (pH 7.5) with 3 mM $MgCl_2$, and 0.5 ml of freshly prepared ONPG in 0.01 M Tris-acetate (pH 7.5) containing 0.01 M $MgCl_2$ in a 16 mm x 100 mm test tube and shaken at 180 RPM for 10 min at 30°C. The β-gal reaction was slowed down by adding 0.3 ml of saturated sodium carbonate and then the resultant colour was immediately measured at 405 nm.

(iv) AP:

After 0 to 18 days of incubation, AP samples were removed from each condition and diluted 100 times with 1.0 M diethanolamine buffer (pH 9.8) containing 0.5 mM $MgCl_2$. Fifty μl of the diluted AP suspension was mixed into a pre-warmed (37°C) 16 mm x 100 mm test tube with 0.5 ml of 15 mM p-npp in diethanolamine buffer and shaken at 180 RPM for 10 min at 37°C. The AP reaction was stopped by adding 0.5 ml of 0.1 M EDTA·2 Na·2 H_2O with the resulting colour being measured at 405 nm.

RESULTS AND DISCUSSION

To test the PVA stabilization effect, each enzyme was suspended in phosphate buffered saline containing 0.1 % (v/v) Micro-O-Protect (PBSM) with or without 5 % PVA of different molecular weights. Micro-O-Protect, which had no effect upon the activity of any of the enzymes, was used as a preservative to prevent microbial degradation of the enzymes. A 5 % concentration of PVA was used in order to maximize its stabilization effect upon the different enzymes. The enzymes were suspended at an appropriate concentration so that, following a final dilution prior to assaying, any loss of activity during incubation at 30°C for HRP, GO, and β-gal, or 40°C for AP was measurable. Since AP is stable at 30°C, it was necessary to increase the incubation temperature to 40°C in order to see a decline in AP activity within a reasonable period of time. Although phosphate ions from the PBSM are known to act as a reversible inhibitor of AP activity, the activity of the AP was measured following a 100 times dilution in diethanolamine buffer to a non-inhibiting phosphate concentration.

Figure 1A shows that HRP was stabilized in the presence of all PVA conditions examined. After 15 days of incubation at 30°C, HRP stored in PVA (MW 9,000-10,000 or 30,000-70,000) retained approximately 80 % of its initial activity as compared to 42 % for HRP stored in PBSM. Since PVA stabilized free HRP as well as antibody-HRP conjugates (Boyd and Yamazaki, 1994), PVA is expected to stabilize conjugates of other enzymes if it is shown to stabilize the corresponding free enzyme. Figure 1B shows that PVA also stabilized GO. GO retained 94-98 % of its initial activity after 7 days of incubation at 30°C in the presence of the various PVA solutions with the exception of PVA (MW 70,000-100,000) in which 84.2 % of its initial activity was retained. These results suggest that PVA should be able to stabilize GO conjugates. Figure 1C demonstrates that the various PVA conditions had no significant stabilization effect upon β-gal. Therefore, PVA may not be useful in the stabilization of β-gal conjugates. Figure 1D shows that none of the PVA conditions examined yielded a stabilization effect upon AP, suggesting that PVA may not be able to stabilize AP conjugates. The stability of free GO in PVA (Figure 1B) can extend the usefulness of this analytical enzyme for glucose assay since a GO solution can be stored at ambient temperature for at least one week. HRP, GO and AP are glycoproteins while β-gal is not. The carbohydrate content of HRP (Shannon *et al.*, 1966) and GO (Tsuge *et al.*, 1975) is approximately 18 % while that of adult bovine AP is approximately 12 % (Besman and Coleman, 1985). Extensive hydrogen bonding between H-atoms of alcohol groups in

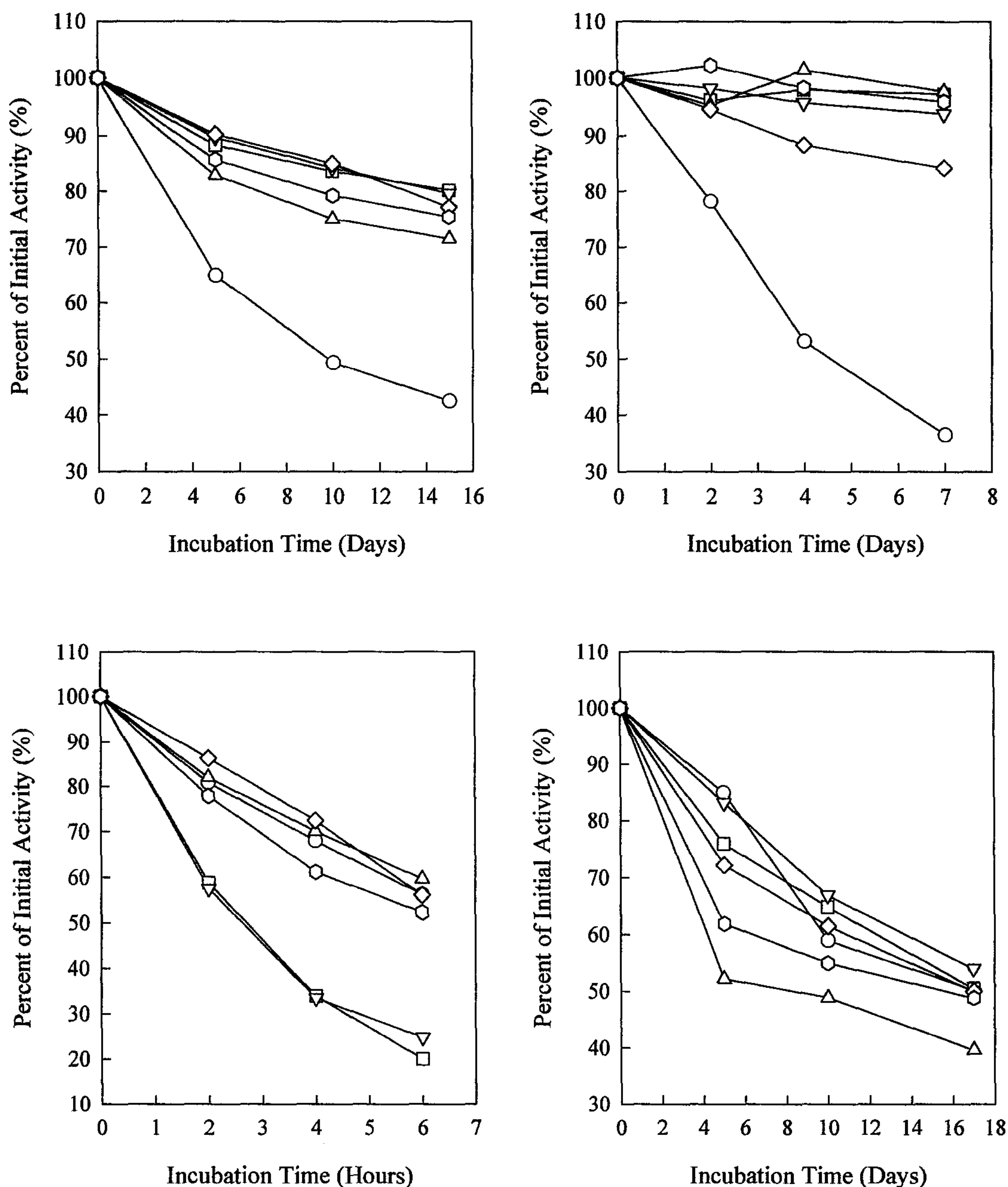

Figure 1: The effect of different molecular weights of PVA upon the activity of HRP (A), GO (B), and β-gal (C) at 30°C, or AP (D) at 40°C. Each enzyme was incubated in PBSM (control) or 5 % PVA in PBSM and then at regular time intervals samples of the different enzyme solutions were assayed for enzyme activity as described in Methods. The average absorbance for each enzyme at each time interval is plotted as a percent of initial activity at time 0 ± standard deviation (n = 6). Symbols: ○, PBSM; □, PVA (MW 9,000-10,000); ▵, PVA (MW 13,000-23,000); ▿, PVA (MW 30,000-70,000); ◇, PVA (MW 70,000-100,000); ⬡, (MW 124,000-186,000).

PVA and O-atoms of carbohydrate groups in HRP and GO may be responsible for the observed HRP and GO stabilization by inhibiting the formation of non-functional conformations.

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