Study of the Thermal Stability of Glucose Oxidase in the Presence of Water-Soluble Polymers

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ABSTRACT: The thermal stability of glucose oxidase (GOD) in solutions containing water-soluble hydrolyzed polyacrylonitrile (HPAN) and polyoxyethylene (POE) was studied as a function of time and temperature between 28 and 60°C. The results were compared with the thermal stability of GOD in solutions without polymers. The polymers studied were found to increase the enzyme thermal stability. The influence of the concentration of the water-soluble polymers on enzyme thermal stability was also studied. The best protection effect on enzyme thermal stability had 3 wt % solution of HPAN and 1 wt % solution of polyoxyethylene. Solutions with higher concentrations led to a quick deactivation of the enzyme. It was proved that the effect of 3 wt % HPAN solution was stronger than the effect of 1 wt % POE (59.0 versus 52.0%). The thermal transition of the enzyme was studied in both the presence and the absence of HPAN by DSC. The melting temperature of GOD in the presence of HPAN was shifted to an 11°C higher value. This sustained the supposition that HPAN increases the thermostability of GOD. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 90: 1393–1397, 2003

Key words: glucose oxidase; hydrolyzed polyacrylonitrile; polyoxyethylene; thermal properties; stabilization

INTRODUCTION

Some of the most important characteristics of an enzyme are its catalytic activity and stability. These can be enhanced by adding various compounds (e.g., glycerin, sugars). The additives form numerous hydrogen bonds with the amino acid residuals, thus limiting the spontaneous or heat-induced denaturation of the enzymes.[1]

A number of recent publications have reported that the addition of polymers (e.g., ethylcellulose, polyethyleneglycol, polyvinylpyrrolidone, polystyrene) enhances the stability of the enzymes.[2–8] It has been supposed that the interaction between the enzyme and polymer results in formation of complexes that improve enzyme stability.

The effect of polyoxyethylene (POE) on the activity and thermal stability of some enzymes is well known.[1,9–11] It has been found to depend on the molecular weight of POE.[12,13] It is considered that the higher thermal stability of the enzyme in the presence of POE is attributed to the interaction between the enzyme and POE.

There are no publications available, however, on the effect of water-soluble hydrolyzed copolymer of acrylonitrile–methylmethacrylate–sodium vinylsulfonate on the activity and thermal stability of enzymes.

The aim of the present study was to study the effects of hydrolyzed polyacrylonitrile (HPAN) on the thermal stability of glucose oxidase (GOD) and compare the obtained results with that for POE. GOD was selected for the experiments because of its widespread use and importance as a reagent in medical diagnostics, where the enzyme should be stable enough, and its cost-effective use.

EXPERIMENTAL

Chemical reagents

A copolymer of acrylonitrile (91.03 wt %)–methylmethacrylate (7.3 wt %)–sodium vinylsulfonate (1.4 wt %), supplied by Lukoil-Neftochim Co. (Bulgaria), was used for the experiments. The water-soluble copolymer used for the modification of GOD was prepared by hydrolization of the copolymer with NaOH (Fluka, Buchs, Switzerland). POE (molecular weight of 1×10^5 Da) was produced by Chimko Co. (Bulgaria). The GOD used (from Aspergillus niger) had specific activity of 21 U/mg protein and molecular weight of 1.5×10^5 Da (Fluka).

Preparation of water-soluble HPAN

NaOH (100 g) and copolymer (100 g) acrylonitrile–methylmethacrylate–sodium vinylsulfonate were

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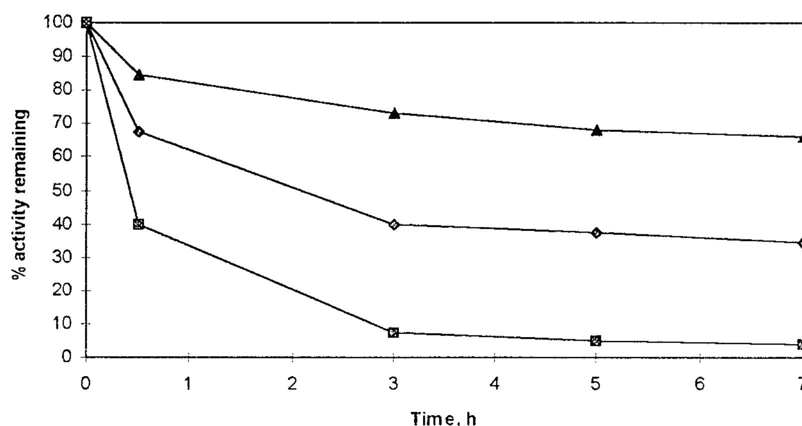

Figure 1 Effect of time on the enzymatic activity of glucose oxidase at different temperatures: (▲) 28°C; (◆) 45°C; (■) 60°C. Enzyme concentration: 0.0008 mg/mL.

mixed in 100 mL distilled water. The mixture was heated for 6 h at 80°C while stirring with mechanical stirrer. The hydrolyzate obtained was neutralized to pH 7.

Preparation of GOD modified with HPAN and POE

Water-soluble HPAN and POE were added to 0.1*M* phosphate-buffer solution (pH 5.8) containing 0.0008 mg/mL GOD. The amount of HPAN was varied from 1 to 36 wt %, whereas that of POE, from 0.2 to 1.5 wt %.

Enzyme analyses

The free and modified GOD activities were determined by a spectrophotometrical method (Specol 11; Carl Zeiss Jena, Germany)[14] involving peroxidase and *o*-dianisidine. The hydrogen peroxide, obtained as a byproduct of β-D-glucose oxidation, was reduced by peroxidase and the chromogen (*o*-dianisidine) was oxidized and measured spectrophotometrically at 460 nm.

Determination of the thermal stability of free and modified GOD

The solutions with free and modified GOD were tempered in a water thermostat at temperatures from 28 to 60°C for 7 h. Samples were taken at certain periods and the specific activity of GOD was determined as described above.

Calorimetric analyses of free and modified GOD

The calorimetric analyses were performed with a Perkin–Elmer DSC-2 (Perkin Elmer Cetus Instruments, Norwalk, CT) at a heating rate of 10 K min^{-1} and with a scanning region from 20 to 100°C. Samples of the solutions (pure 3 wt % HPAN, pure GOD, and GOD modified with 3 wt % HPAN) were sealed in aluminum crucibles. The weights of the samples were measured gravimetrically after drying them at 100°C for

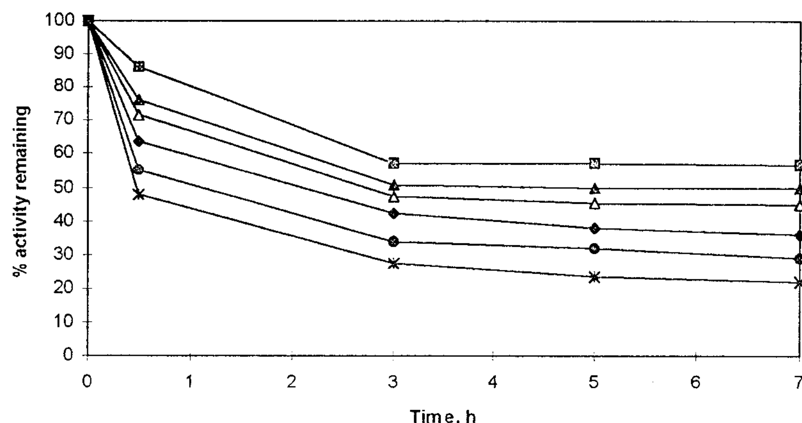

Figure 2 Effect of time on the enzymatic activity of unmodified (◆) and modified glucose oxidase with hydrolized PAN at different concentrations: (△) 1 wt %; (■) 3 wt %; (▲) 6 wt %; (●) 24 wt %; (∗)36 wt %. Enzyme reaction temperature: 45°C.

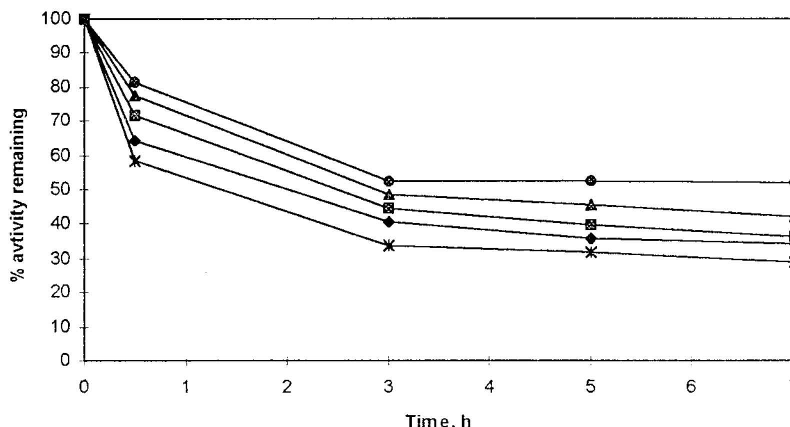

Figure 3 Effect of time on the enzymatic activity of unmodified (◆) and modified GOD with POE at different concentrations: (■) 0.2 wt %; (▲) 0.5 wt %; (●) 1 wt %; (*) 1.5 wt %. Enzyme reaction temperature: 45°C.

24 h. The accuracy of the measurement of the temperature transition was ±1°C.

RESULTS AND DISCUSSION

The thermal inactivation of GOD was studied in the temperature range from 28 to 60°C for periods from 0 to 7 h (Fig. 1). As could be expected, the inactivation rate increased with temperature. At 28°C, only 16% of enzyme activity was lost after 0.5 h incubation, whereas at 60°C the inactivation was rapid and the enzyme activity decreased by more than 60%.

The effect of water-soluble polymer (HPAN) on the thermal stability of GOD was investigated. HPAN was prepared by total hydrolysis of PAN with molecular weight 52 kDa. Because of the alkali hydrolysis, the polymer chains were broken and the nitrile groups were hydrolyzed to amino groups (~ 2%) and carboxylic groups (~ 60%). The molecular weight of the hydrolyzate obtained was determined viscosimetrically to be 15 kDa. The low molecular weight and large amount of carboxylic groups gave the hydrolyzate high solubility in water.

The effect of HPAN concentration on the thermal stability of GOD was studied at 45°C for 7 h (Fig. 2). For comparison, the thermal stability of GOD in phosphate-buffer solution without HPAN was also investigated. Obviously, the higher stability showed GOD in the presence of 3 wt % HPAN solution. The inactivation of GOD in this solution was 41.0%. The inactivation of the comparative GOD without HPAN was significantly higher at 65.0%.

At higher concentrations of HPAN (24 and 36 wt %) the inactivation was faster than that in solutions with lower concentrations (1, 3, and 6 wt %). After 7 h it was found to be 72.0 and 78.0%, respectively. Therefore, the addition of HPAN solution with concentrations up to 6 wt % thermally stabilized the enzyme. The best results were obtained with 3 wt % solution of HPAN where GOD loss was 41.0% of its initial activity from 3 to 7 h, which was 1.59 times lower than the loss of enzyme activity in solution without HPAN (65.0%).

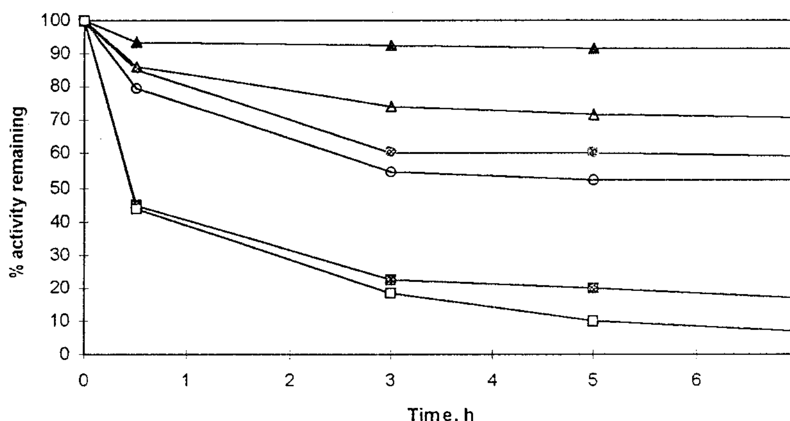

Figure 4 Effect of time on the enzymatic activity of GOD modified with 3 wt % hydrolized PAN at different temperatures: (▲) 28°C; (●) 45°C; (■) 60°C, and 1 wt % POE: (△) 28°C; (○) 45°C; (□) 60°C.

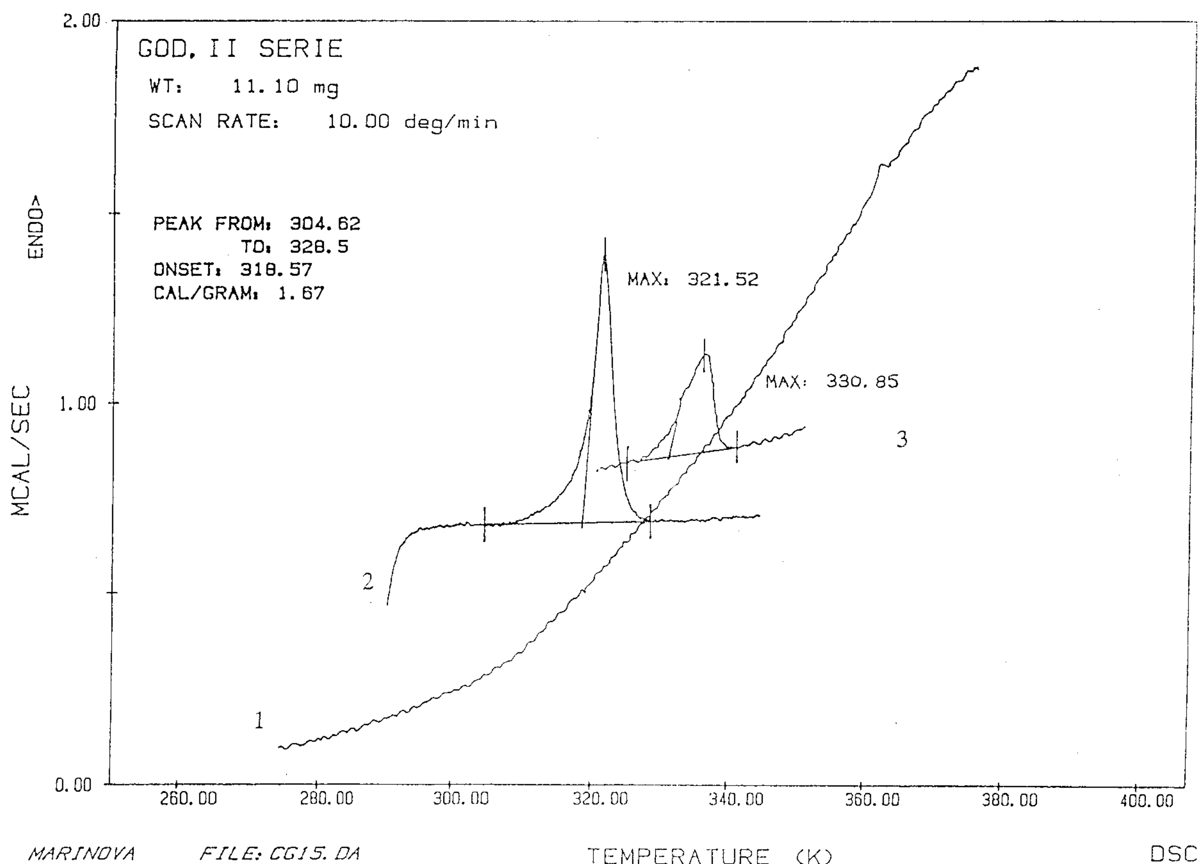

Figure 5 Thermograms of hydrolyzed PAN (1), GOD (2), and GOD with added hydrolyzed PAN (3).

It is well known that POE is a good enzyme stabilizer. Therefore, its effect on the thermal stability of GOD was also studied for comparison with the water-soluble hydrolyzed PAN. The kinetics of thermal inactivation of GOD was studied at the same temperature (45°C) in the presence of different concentrations of POE (Fig. 3). The molecular weight of POE used was 1×10^5 Da because, as reported by other investigators,[12] good stabilization of the enzyme can be achieved with POE of molecular weight at least 4000 and the stability increases with the molecular weight of POE.

It can be seen from Figure 3 that the addition of POE, in concentrations up to 1 wt %, improved the enzyme stability compared to that of free enzyme. The best results were obtained in the presence of a 1 wt % solution of POE (at 7 h GOD loss was 48.0% of its initial activity). With 1.5 wt % POE the inactivation of the enzyme was very fast (~ 71.0% at 7 h).

By comparing the results discussed above it can be concluded that better stabilizing effects were observed with 3 wt % HPAN and 1 wt % POE solutions. These two polymers were further compared to find which one exerts the stronger effect. Figure 4 shows the kinetics of thermal inactivation of GOD in the presence of 3 wt % HPAN and 1 wt % POE at 28, 45, and 60°C for 7 h. The enzyme activity loss (%) was determined to be 7.5% at 28°C, 41.0% at 45°C, and 82.0 at 60°C with 3 wt % HPAN. With 1 wt % POE the values measured were 30.0% at 28°C, 48.0% at 45°C, and 93.0% at 60°C (Fig. 4). Obviously, the 3 wt % HPAN solution showed better stabilizing effect than that of 1 wt % POE in all the cases studied.

Probably the better stabilizing effect of the HPAN compared with that of the POE is attributable to formation of chelates between carboxylic groups and amino groups of HPAN and, respectively, NH_2 and COOH groups of the enzyme. It is considered that weaker hydrogen bonds are formed between hydroxyl groups of the POE and the enzyme. The supposed bonding between the GOD functional groups and the HPAN and POE allows us to classify the water-soluble polymers treated with GOD as "modified."

On the other hand, the higher inactivation rate at higher HPAN and POE concentrations (which implied that denaturation of GOD took place) can be explained only as follows: the protein molecule usually unfolds at thermal inactivation. It reveals a new protein surface, and additional bonds between protein functional groups and the polymer, which decreased GOD stability in concentrated solutions of HPAN and POE.

It was important to study the possibility of the enzyme to perform its catalytic function over a wide temperature range (20–60°C) and to determine the optimal temperature (T_{opt}). The enzyme activity was measured for 10 min at pH 5.8. Experiments were carried out with 3 wt % solution of HPAN and 1 wt % solution of POE with which the highest stability of the enzyme was observed. The results were compared with those of nonmodified GOD. The T_{opt} of the native GOD reported earlier[15,16] was 28°C. In the presence of HPAN and POE the optimum was shifted to a higher value (30°C) and, at temperatures higher than 30°C, the enzyme activity was higher than that for polymer without water. It supports the conclusion that the thermal stability of GOD increases in the presence of water-soluble polymer.

Differential scanning calorimetry is useful for studies of biological macromolecules.[9,11] It provides information on the thermal stability of the protein indicated by the transition temperature when a sample of protein is heated and undergoes conformational changes. Figure 5 presents the DSC thermogram of GOD in phosphate-buffer solution with pH 5.8 (Fig. 5, curve 2), HPAN (curve 1), and GOD in phosphate-buffer solution with added HPAN (curve 3). Obviously, GOD underwent a full thermal transition in the temperature interval studied (20–100°C). The DSC thermogram for HPAN (curve 1) shows that this polymer does not have clear temperature transitions. The melting temperature of GOD was measured to be 47°C. The repeated heating of the samples did not produce a transition, probably because GOD had undergone an irreversible change in its conformation (the enzyme was denaturated). The thermogram for GOD with HPAN (curve 3) shows that the melting temperature of GOD increased to 57°C, which once again sustained our supposition that HPAN increases the stability of GOD.

CONCLUSIONS

The water-soluble HPAN and POE under investigation were found to increase the enzyme thermal stability. The best protection effect on enzyme thermal stability was a 3 wt % solution of HPAN and 1 wt % solution of POE, although it was proved that the effect of 3 wt % HPAN solution was stronger than the effect of 1 wt % POE (59.0 versus 52.0%).

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