Polymer Bulletin 53, 63–71 (2004)
DOI 10.1007/s00289-004-0309-6

Polymer Bulletin

The PVA solution structure-change effect for α-amylase specific activation

Takashi Takada, Toshihiro Hirai (✉)

Faculty of Textile Science and Technology, Shinshu University, 3-15-1 Tokida, Ueda City, Nagano, 386-8567, Japan
Tel.: +81-268-21-5405; Fax: +81-268-21-5391

E-mail: tohirai@giptc.shinshu-u.ac.jp (T.Hirai)

Received: 7 September 2004 / Revised version: 25 October 2004 / Accepted: 26 October 2004
Published online: 30 November 2004 –

Summary

The effect of the addition of poly(vinyl alcohol) (PVA) on the α-amylase activity from Bacillus subtilis was investigated. About 500% of spiky activation on the reaction rate was observed in the presence of 0.25wt% PVA in the reaction medium. This activation was induced by the addition of the PVA, and was resulted from a structural-change of the solution due to the PVA. The structure change of the PVA solution was analyzed with a fluorescent dye as a hydrophobic site probe, and the activation was not observed when the polymer-additive was changed from PVA to poly(ethylene glycol) (PEG). We showed that the hydrophobic site structure of the PVA was important for this specific activation.

Introduction

Enzymes are indispensable as catalysts in living systems, and they have been studied extensively in practical applications for many years. The immobilization of enzymes is widely used for industrial applications and in the field of medicine. However, the activity of the immobilized enzymes is usually depressed by the immobilization. The environmental change around the enzymes is one of the reasons of this activity reduction. The conformation and activity of the enzymes are sensitive to the external environmental changes, for example pH, pressure etc. [1-3]. The immobilization support may also cause changes of the environment around the enzyme when it is immobilized by entrapping. Therefore, the relationship between the immobilization matrix and the enzymes is an important factor. However, there have been very few studies of this relationship because of the complexity of the phenomena involved, and the difficulty in the detection of the weak interaction among the species by conventional methods.

On the other hand, a study of interactions among enzymes and other molecules as environmental control factors requires several kinds of investigations. One of the approaches is to investigate the effect of addition of low molecular-weight molecules to enzymes. For example, the effects of adding low molecular weight polyols [4] or surfactants [5,6] have already been published.

It is very important to clarify the weak interaction between an enzyme and the polymer support from the viewpoint of the advancement of an efficient immobilization technique of enzymes, since reduction of enzyme activity can be minimized by providing a gentle and optimized immobilization environment. However, it is difficult to detect the weak interaction in the conventional immobilization conditions. Therefore, we investigate the effect of the polymer addition on the enzyme activity. In the course of this study, we came across a specific behavior of the activity enhancement in the solution of very low concentration of a polymer, poly(vinyl alcohol) (PVA). This system was turned out to be adequate for the detection of the weak interaction or the slight environmental change around the enzymes. The dilute solution of polymer, in our case PVA aqueous solution, can be expected as a model of polymer support, in which we can investigate the environmental change of the enzyme (in this paper Amylase) by concentrating on the enzyme-polymer interaction excluding other complicated factors.
In general, the presence of a polymer in the reaction medium increases its viscosity, depresses substrate diffusion and causes a depression of the enzyme-reaction. However, this effect can be avoided by using a polymer in a super-diluted solution of the reaction medium. The effect of environmental-changes on enzymes was examined by changing the concentration of the dilute polymer solution.
We tried to analyze the effect of the addition of PVA in the enzyme reaction medium by following the enzyme reaction kinetics. Changes in activity were usually induced by a change in the enzyme conformation, so we also tried to measure the changes in the enzyme conformation by observing the CD spectrum. Changes in the activity and conformation of the enzymes were induced by structural changes of the solution containing the PVA molecules in the reaction medium. The changes in the solution structure caused by the PVA were examined by fluorescence analysis using a fluorescent dye as a hydrophobic site probe. The specific properties of the PVA were compared with those of PEG. The PEG are similar to PVA in chemical composition, but their chemical structures are different.
We used α-amylase as a model of an immobilized enzyme. Amylases are among the most extensively investigated enzymes, and are one of the most useful enzymes in several industrial and medical fields.
The reaction of enzymes in polymer solutions could be applied for use in bioreactors and in intra-cellular reaction analysis. Additionally, the environmental sensitivity of biomacromolecules suggests their application as the indirect probe of the structure of a dilute polymer solution.

Materials and Methods

Enzyme and substrate

α-Amylase (from Bacillus subtilis) was purchased from Wako Pure Chemicals Co. (Osaka, Japan) and was used without further purification. Soluble starch and β-D-glucose were purchased from Sigma Chemical Co. (St. Louis, USA).

Polymers

PVA and PEG were used as the additives.
PVA (Kuraray PVA-117, DP=1700) was supplied from Kuraray Co., Ltd. (Osaka, Japan). The PVA was extracted with warm methanol for 20 hours or more with a Soxhlet extractor after complete saponification, (according to the procedure of JIS

K-6726[7]). The saponified PVA was re-precipitated at least 3 times with methanol and was then used for the following experiments.
PEG (DP=450) was purchased from Wako Pure Chemicals Co. (Osaka, Japan), and was used without further purification.

Other reagents

The reagent 8-anilinonaphthalene-1-sulfonic acid (ANS) was used for fluorescence measurements to analyze the structure of the PVA solution. The ANS was purchased from Wako Pure Chemical Co. (Osaka, Japan) and was used without further purification.
The other reagents were commercially available extra-pure grade chemicals and were used without further purification.

Determination of α-amylase activity

5.0mM acetic acid buffer solutions (pH 6.0, ionic strength (μ) = 0.15, adjusted with $CaCl_2$ and NaCl) containing a prescribed amount of soluble starch and PVA together were prepared as substrate solutions. The PVA concentrations were between 0.0 and 0.5wt%, and the substrate concentrations were 0.75 to 5.0mg/ml. 50 ml of the substrate solution was put into a flask and was maintained at 25°C. Similarly, an enzyme solution was maintained at 25°C. The reaction was started by adding 5.0 ml of the enzyme solution to the substrate solution. An aliquot of the reaction solution was extracted after a prescribed reaction time, and the reaction products were measured by the Somogyi-Nelson method [8, 9]. The colorimetric determination was carried out using a HITACHI U-3210 spectrophotometer.
Calibration curves were obtained from solutions containing prescribed amounts of PVA and β-D-glucose. A calibration curve for β-D-glucose was determined for each PVA concentration, and the α-amylase reaction was analyzed in the usual manner for enzyme kinetics [10].

Fluorescence measurements

The structural-change of the PVA solution was examined using florescence measurements. ANS was used as a probe [11] at an excitation wavelength of 360nm. The fluorescence spectra were measured using a Shimadzu RF-5000 fluorescence spectrophotometer. ANS was added to the PVA solution of prescribed concentration, with and without α-amylase and substrates. The range of concentrations of PVA addition was between 0.0 and 0.75wt%.

CD measurement

The conformational-changes of the α-amylase were examined from the CD spectra. The samples used for CD measurement were the same as those used for activity measurements. For this measurement, the samples did not contain a substrate. The concentration range of PVA addition was between 0.0 and 0.5wt%. The CD spectra were measured on a JASCO spectropolarimeter, Model J-600, installed with a standard analysis program. The spectra were recorded with a quartz cell of 0.2-mm path-length, over the wavelength range from 200 to 240 nm.

Results and Discussion

The effect of PVA addition for α-amylase activity

In this work, we tried to evaluate the effect of PVA addition on the α-amylase enzyme reaction. Generally speaking, the presence of a polymer in the reaction medium depresses substrate diffusion and causes a depression of the enzyme-reaction. However, in this research the addition of PVA caused an increase in activity; Figure 1 shows the time-course of the enzyme reaction in the presence or absence of PVA. It was clearly observed that the reaction rate and the amount of product were increased when PVA was added. This activation was also observed at other PVA concentrations and at other substrate concentrations.

The depression of the enzyme reaction cannot be shown under the experimental conditions employed in this research, for the reasons described below. The concentration of the PVA was very dilute, so the PVA globules dispersed do not interact with each other [12] and the PVA molecules did not unfold in the reaction medium. The folded molecules formed globules. Therefore, the diffusion of the various reagents was not affected by the PVA globules. These properties were observed when the PVA molecules were perfectly saponified.

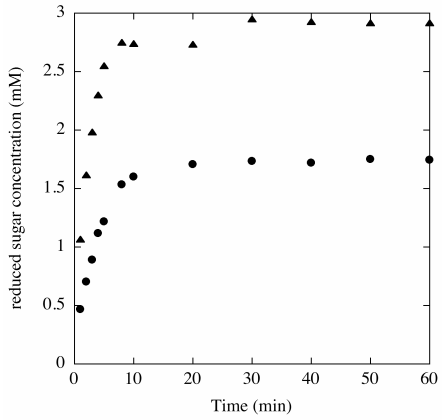

Figure 1. The time-course of α-amylase reaction in the absence (circle) and presence (triangle:1.00wt%) of PVA. ; [S_0]=2.5mg/ml; [E_0]=24.5μg/ml; at 25°C.

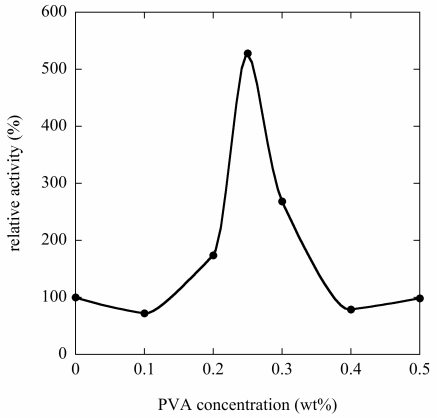

Figure 2. The relation between initial reaction rate and PVA concentration. ;[S0]=2.5mg/ml; [E0]=24.5μg/ml; at 25°C.

The relationship between the relative reaction rate and the PVA concentration is plotted in Figure 2. The α-amylase was activated when PVA was added in almost all regions. Figure 2 shows that the change in the initial reaction rate is specific and non-linear with respect to the PVA concentration, and that the initial reaction rate shows a peak at a PVA concentration of 0.25wt%. Most importantly, the rate at 0.25wt% is about 5 times to that of the native state or in the absence of PVA. This specific activation at 0.25wt% PVA was observed for all substrate concentrations, suggesting that the specific activation originates from the PVA. This result suggested the influence of PVA in changing the structure of the solution, since this specific activation only occurred for changes in the PVA concentration. The specific activity change was nonlinear, but the

PVA concentration change was linear. The reason for the difference between the activation and the amount of PVA that was added were due to the changes in the structure of the PVA solution. Similar change is suggested in other cases. For example, the addition of a surfactant above and below the cmc (critical micelle concentration) for the enzyme reaction caused changes in the enzyme activity that was dependent on the cmc [5]. This study relates closely to our system. However, in our reaction system, the enhancement of the activity is much larger than the reported one. We are not aware of a similar specific, enormous non-linear increase in activation in other research.

The effect of PVA addition on α-amylase conformation, as measured from the CD spectrum

The specific activation of α-amylase by the addition of PVA was observed. The activity change was usually accompanied by a conformational-change of the enzymes. Therefore, we tried to measure the CD spectrum. The CD spectrum is widely used in the field of protein analysis for long time. The CD spectrum for a solution containing only PVA did not show any absorption. Figure 3 shows the CD spectra of α-amylase with and without PVA. The spectra of almost all of the PVA-added samples were similar to the spectrum without PVA (Figure 3a). However, specific absorptions were observed at 0.25 and 0.40wt% PVA addition (Figure 3b).

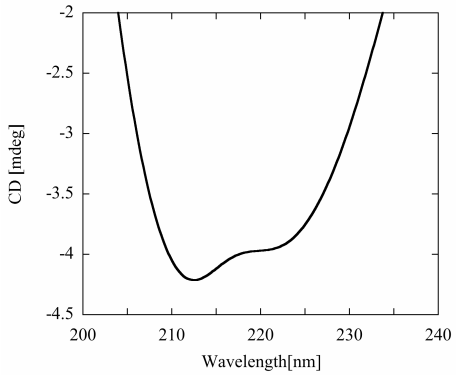

Figure 3a. The ultraviolet CD spectrum of native α-amylase.

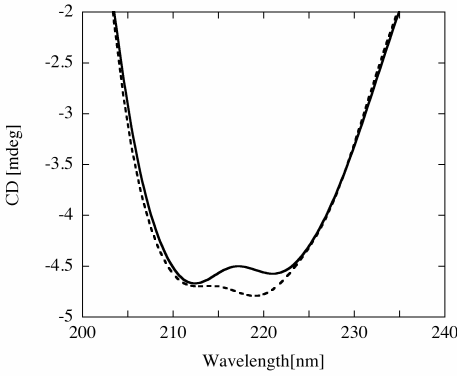

Figure 3b. The ultraviolet CD spectrum of α-amylase with the PVA. The solid line and dashed line show the 0.25 and 0.40wt% PVA addition, respectively.

These specific absorptions suggest the presence of α-amylase conformational change of some kind. The CD spectra change at 0.25wt% PVA was attributable to the α-amylase activation. However, it is not for the 0.40wt% PVA. This reason can be considered as below. Generally, the CD absorption peak at 218nm (correspond to 0.40wt% PVA) is due to the beta structure [13]. And, the absorption at 218nm is well known to be sensitive to the external environment [14]. The CD change of 218nm in our study can be expected as the result of the influence by the external environment. However, the further details on α-amylase conformation change requires more direct structure analysis with other method which is not available here.

Structural analysis of the PVA solution by fluorescence measurements using a hydrophobic site probe

The existence of a specific activation of α-amylase by the addition of PVA was clear from the activity measurements. The structure of the PVA solution was important in terms of both the activity and structural changes in the α-amylase. The structural-analyses of PVA solutions have already been investigated extensively [15, 16]. However, these studies differ from our work in terms of the PVA concentration range and the particular measurement method used. High concentration samples were usually required for the measurements in order to obtain high-reliability data. The usual measurement techniques (e.g. light scattering) could not obtain highly reliable data at such low concentrations as were employed in this study.

We then tried to analyze the structure of the PVA solution using fluorescence measurements [17]. The fine environment of the PVA solution was examined using ANS as a fluorescence hydrophobic site probe. By being absorbed on the hydrophobic site, ANS decreased its fluorescence maxima, and increased its fluorescent intensity. The fluorescent properties changes if the PVA solution forms domains with some hydrophobicity. The influence of the PVA concentration on the fluorescence maxima and on the differential fluorescence intensity (delta F) of ANS is shown in Figure 4. The maxima decreased and the intensity increased, suggesting that ANS was absorbed in the hydrophobic domain of the PVA. This change was possible because the PVA can form a little hydrophobic domains. However, the changes are not linear, and they both show a peak at 0.25wt% PVA. Changes of this nature were also observed when substrate or enzymes were present in the PVA solution (Figure 5).

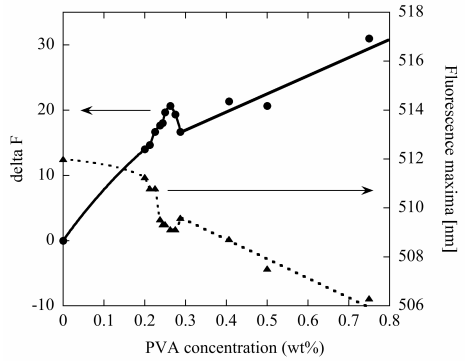

Figure 4. The influence of PVA concentration on fluorescence maxima and difference fluorescence intensity (delta F) of ANS at pH6.0. : ANS=0.05mM ; excitation=360nm.

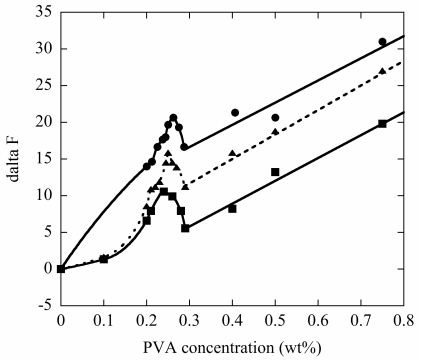

Figure 5. The influence of PVA concentration on difference fluorescence intensity (delta F) of PVA solution (circle), PVA with α-amylase (triangle; [E]=24.5μg/ml) and PVA with substrate (square; [S]=2.5mg/ml) at : ANS=0.05mM ; excitation=360nm.

It was suggested that the changes in the structure of the solution could be mainly attributed to the PVA concentration, since the other factors did not have a pronounced effect. The result of the fluorescence measurements showed that hydrophobic site formation and breakdown occurred at PVA concentrations near to 0.25wt%. We explained this change as described below. The distance among the PVA globules

decreased when the PVA concentration increased. Usually, the hydrophilic sites of the PVA globule are on the outside in water solution. In these circumstances, the inside of the globule is comparatively hydrophobic. However, the hydrophobic sites can move to the outside when the distance between the PVA globules is decreased and the PVA globules begin to merge with each other. This change in the conditions of PVA hydrophobic sites affected the environment of the α-amylase, and the α-amylase structure and activity were changed (Figure 6).

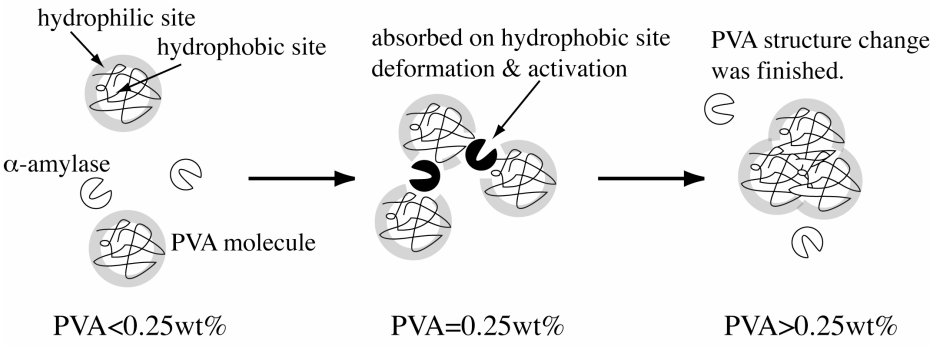

Figure 6. The illustrations of the α-amylase activation mechanisms by PVA molecules.

This is similar in the case of the addition of a surfactant to the enzyme reaction system [6].

The difference of PVA and PEG in the effects on the α-amylase reaction

The results discussed above show that the effects on α-amylase activity and structure were caused by a change of a hydrophobic domain in the PVA solution. We therefore tried to confirm the effects of the hydrophobic condition using PEG. PEG is similar to PVA in terms of the compositional-formula of the monomer unit; however it does not have a similar structural-formula or other properties (e.g. solubility). Figure 7 shows the effects of PEG addition on the α-amylase reaction, compared with the addition of PVA. The results show that the α-amylase activity did not change when PEG was added.

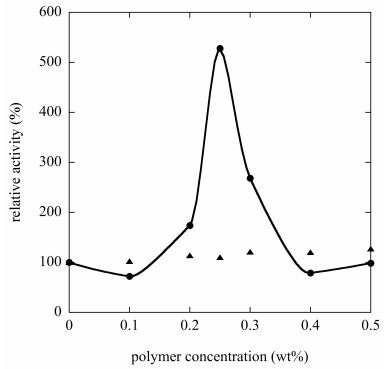

Figure 7. The relation between initial reaction rate and PVA (circle) and PEG (triangle) concentration ; $[S_0]$=2.5mg/ml; $[E_0]$=24.5μg/ml; at 25°C.

The reasons for this result are described below. The PEG is not crystalline, and the molecules are dispersed or solubilized quickly into aqueous solution and the molecular chains are unfolded [18]. The difference from PVA is remarkable. The hydrophobic sites of the PVA were aggregated when the PVA dissolved in water. The aggregate structure changes with PVA concentration non-linearly; however the PEG molecules did not aggregate.
The small and dispersed hydrophobic site formed by the PEG was not sufficient to change the α-amylase structure (Figure 8). The difference in the condition of this hydrophobic site caused the difference in α-amylase activity.

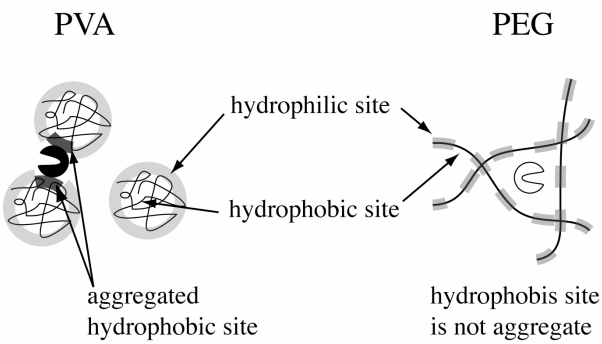

Figure 8. The illustrations of the solution structure difference between PVA and PEG.

Conclusion

The above results confirm that the change in α-amylase activity occurred due to a change in the hydrophobic structure of the PVA aqueous solution. The 500% enhancement of the activity showed a spiky profile. Activity showed a maximum value at 0.25wt% PVA. This change in activity was specific to the condition of the hydrophobic site of the PVA. This result suggests that a delicate control of the enzyme environment can lead to an enhancement of the enzyme activity, and suggest that the method can be applied for clinical inspection and as a high performance bioreactor designing. This research was also of interest from the viewpoint of mimicking an artificial inter-cellular environment.

References

1. Klaus Mosbach Ed. (1977) Methods in Enzymology 44, Academic Press, New York
2. Ichirou Chibata Ed. (1986) Koteikaseitaishokubai. Kodansha, Tokyo
3. Robert K.Murray, Daryl K.Granner, Peter A.Mayes and Victor W.Rodwell. (1993) Harper's Biochemistry 23 Ed.,APPLETON & LANGE ,Conneticut
4. D.Combes, M.Graber and W.N.Ye, Stabilizing effect of polyhydric alcohols, Ann. NY Acad. Sci. 613 (1990) 559-563.
5. A.Tanaka and E.Hoshino, Thermodynamic and activation parameters for the hydrolysis of amylose with Bacillus α-amylase in a diluted anionic surfactant solution, J.Biosci. Bioeng. 93 (2002) 485-490.
6. M Hirai, R Kawai-Hirai, T Hirai, and T Ueki, Structural change of jack bean urease induced by addition of surfactants studied with synchrotron-radiation small-angle X-ray scattering Eur. J. Biochem. 215 (1993) 55-61.
7. Japanese Industrial Standard JIS K-6726

8. Somogyi M., Notes on sugar determination, J.Biol.Chem. 195 (1952) 19-23.
9. Nelson N., A Photometric adaptation of the Somogti method for the determination of glucose, J.Biol.Chem. 153 (1944) 375-380.
10. Irwin H., Segel, (1975) Enzyme Kinetics, John Wiley & Sons Inc, New York.
11. GR Penzer, 1-anilinonaphthalene-8-sulphonate. The dependence of emission spectra on molecular conformation studied by fluorescence and proton-magnetic resonance, Eur J Biochem, 25 (1972) 218-228.
12. K.Nagano, S.Yamane and K.Toyoshima, Poval, Kobunshikankokai, Kyoto JAPAN, 1970.
13. Yang JT, Wu CSC, Martinez HM, Calculation of protein conformation from circular dichroism., Methods in Enzymology, 130 (1986) 208-269.
14. S.Brahms and J.Brahms, Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism, J.Mol.Biol. 138 (1980) 149-178
15. M.Ohkura, T.Kanaya and K.Kaji, Geration rates of poly(vinyl alcohol) solution, Polymer 33 (1992) 5044-5048.
16. K.Sho and S.Kawaguchi, SEC-MALS characterization of aggregation behavior of poly(vinyl alcohol) in water, Kobunshi Ronbunshu 60 (2003) 300-304.
17. K. A. Al-Hassan and M. A. El-Bayoumi, Large edge-excitation red shift for a merocyanine dye in poly(vinyl alcohol) polymer matrix, J. Polym. Sci. B., 25 (1987) 495-500.
18. J.Milton Harris, (1992) Poly(ethylene glycol) chemistry, Plenum Press, New York.