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Characterization of polymer–enzyme complex as a novel biocatalyst for nonaqueous enzymology

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Abstract

The optimal methodology to prepare the novel modified enzyme, polymer–enzyme complex, was developed to give a high catalytic activity in anhydrous organic media. The complex was prepared by mixing an enzyme and various polymers in various buffer solutions, and subsequently lyophilizing them. Optimal preparation conditions were obtained when poly(ethylene glycol) (PEG) 4000 was used as a modifier of α-chymotrypsin for the transesterification of Ac-L-PheOEt and 1-propanol in anhydrous isooctane. Additionally, we found that poly(ethylene glycol) and poly(vinyl pyrrolidone) gave a polymer–enzyme complex that had a high catalytic activity in organic media. This strongly suggested that the amphiphilic nature of polymers was important to prepare the polymer–enzyme complex in an aqueous buffer solution, and subsequently used in an organic media. We finally succeeded in preparing the poly(vinyl pyrrolidone)–α-chymotrypsin complex which showed ca. 15,000-fold higher activity than native α-chymotrypsin in anhydrous isooctane under the same preparation conditions.

Keywords: Polymer–enzyme complex; Enzyme modification; Organic solvent; α-Chymotrypsin; Activation

1. Introduction

The high catalytic activity and substrate specificity of enzymes make their use a rapidly growing field in organic chemistry. The use of organic solvents as reaction media has dramatically increased the diversity of enzyme-catalyzed reactions [1–3], because there are numerous potential advantages in employing enzymes in organic media, such as an increased solubility of nonpolar substrates and an enhanced thermal stability of enzymes. A pervasive problem is the much lower (generally several orders of magnitude lower) activity of native enzymes "suspended" in anhydrous organic solvents than in water, although numerous enzymes show their catalytic activity in a vast variety of organic solvents. It is, thus, of practical importance to discover ways to activate enzymes in organic media. One of the most effective ways to activate enzymes in organic media is their solubilization. A number of modified enzymes which were soluble in organic media have been proposed to date, including enzymes chemically modified with polymers [4,5] and physically coated with surfactants [6–14]. These modified enzymes, however, have inherent drawbacks. The chemical modification with polymers using laborious procedures leads to the loss

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of enzyme activity, while the physical modification with surfactants results in the contamination of the product with the surfactant of which molecular weight is low. On the other hand, the noncovalently-formed complex of an enzyme with polymers such as alkylated poly(ethyleneimine) [15], sugar-based polymers [16], polystyrene [17], poly(ethylene glycol) (PEG) [18] with a high molecular weight (30–100 kDa) has been proposed as an alternative modified enzyme which showed a catalytic activity in organic media. The excess adsorption of polymers with high molecular weights on the enzyme surface, however, may result in preventing the contact of the enzyme with substrates, thereby the activity of the enzyme preparations was not so high. In one case, the molecular weight of a polymer which coated an enzyme was relatively low (5 kDa), while the polymer/enzyme ratio was very high (3500) [19], resulting in low enzyme activity. We thus named this enzyme preparation "polymer-coated enzyme", not "polymer–enzyme complex" as stated in their papers. To overcome these defects, it is an attractive strategy to use a small amount of modifiers to activate enzymes in organic media.

We recently proposed a novel soluble biocatalyst "polymer–enzyme complex", i.e. the noncovalently-formed complex of an enzyme with polymers, of which the molecular weight was relatively low. It was catalytically active in organic media even when the molar ratio of the polymer/enzyme in the preparation stage was only a unity [20]. We found that the molar ratio of the polymer/enzyme in the preparation stage governed the activity of the polymer–enzyme complex [20,21]. In addition, we recently found a boric acid–NaOH buffer was useful for the activation of the polymer–enzyme complex, and succeeded in preparing the PEG–α-chymotrypsin complex which showed ca. 6000-fold higher activity than native α-chymotrypsin in anhydrous isooctane [22]. These findings suggested that the polymer–enzyme complex was an effective biocatalyst for nonaqueous enzymology. The optimal methodology for its preparation was, however, not clarified. In this article, we aimed to characterize the properties of the polymer–enzyme complex as a novel biocatalyst for nonaqueous enzymology. α-Chymotrypsin was used as a model enzyme. Various factors in the preparation stage of the polymer–enzyme complex, such as the enzyme concentration, the molar ratio of the polymer/enzyme, pH, the nature and concentration of buffers, and the nature of polymers, were optimized to give a high catalytic activity in anhydrous organic media.

2. Experimental

2.1. Chemicals

α-Chymotrypsin (E.C. 3.4.21.1) from bovine pancreas was purchased from Sigma (Type II, C4129) and used without further purification. *N*-Acetyl-L-phenylalanine ethyl ester (Ac-L-PheOEt) and *N*-acetyl-L-phenylalanine (Ac-L-Phe) were also obtained from Sigma. *N*-Acetyl-L-phenylalanine propyl ester (Ac-L-PheOProp) was synthesized and purified in our laboratory. PEG 1000, 4000, 20,000 and 70,000 with respective average molecular weights of 1, 3, 20 and 70 kDa were purchased from Wako Pure Chemical Industries Ltd. (Japan). Poly(vinyl pyrrolidone) with an average molecular weight of 40 kDa, poly(tetramethylene oxide) with an average molecular weight of 1.9–2.1 kDa, poly(vinyl alcohol) with an average molecular weight of 22 kDa, dextran with an average molecular weight of 60–90 kDa and poly(vinyl butyral) with an unknown molecular weight were also obtained from Wako Pure Chemical Industries, Ltd (product number for poly(vinyl butyral): 169-16575). Ethyl cellulose with an ethoxy content of 48% and viscosity of 10 cP (5% solution in 80/20 toluene/ethanol mixture), polystyrene with an average molecular weight of 250 kDa and poly(vinyl formal) with an unknown molecular weight were purchased from Kanto Chemical Co. Ltd. (Japan, product number for poly(vinyl formal): 32284-32). Poly(vinyl chloride) with an average molecular weight of 62 kDa was obtained from Showa Chemical Co. Ltd. (Japan). *N-trans*-Cinnamoylimidazole was obtained from Aldrich Chemical Co. (USA). All other reagents were of analytical grade and purchased from Kokusan Chemical Works (Japan).

2.2. Preparation of the polymer–enzyme complex catalytically active in organic media

Polymer–enzyme complex was prepared by mixing an enzyme and various polymers in various buffer solutions. The protocol of preparing the polymer–enzyme

complex was the same as that previously reported [20,21]. The buffer agents used were potassium dihydrogenphosphate (KH_2PO_4)–dipotassium hydrogenphosphate (K_2HPO_4), KH_2PO_4–NaOH, boric acid (H_3BO_3)–NaOH, H_3BO_3–sodium carbonate (Na_2CO_3), acetic acid ($C_2H_4O_2$)–sodium acetate ($NaCH_3CO_2$), H_3BO_3–sodium tetraborate ($Na_2B_4O_7$), $Na_2B_4O_7$–HCl, potassium dihydrogencitrate ($KH_2C_6H_5O_7$)–$Na_2B_4O_7$, succinic acid ($C_4H_6O_4$)–$Na_2B_4O_7$, KH_2PO_4–$Na_2B_4O_7$, disodium hydrogenphosphate (Na_2HPO_4)–KH_2PO_4, Na_2HPO_4–citric acid ($C_6H_8O_7$), $KH_2C_6H_5O_7$–NaOH, Tris–HCl, potassium hydrogenphthalate ($C_8H_5KO_4$)–NaOH and $C_4H_6O_4$–NaOH. In all the experiments, the buffer concentration was determined only by the components listed above (the small amounts of NaOH and HCl were used to adjust pH when it was slightly different from what we desired). Ru et al. recently reported interesting phenomena that the co-lyophilization with simple inorganic salts such as KCl at a very high concentration (>90 wt.%) enhanced the activity of native enzymes in organic media [23,24]; however, we did not use such simple inorganic salts to prepare the polymer–enzyme complex. The enzyme concentration, molar ratio of the polymer/enzyme, pH of the buffer solution, and the concentration of buffers were 0.1–10 g/l, 1–100, 4–10 and 0–0.25 mol/(g-enzyme), respectively. The mixture of an enzyme and a polymer in a buffer solution (30 ml) were lyophilized under reduced pressure for 24 h. For comparison with the polymer–enzyme complex, lyophilized native α-chymotrypsin was obtained by lyophilizing from an aqueous KH_2PO_4–K_2HPO_4 buffer solution (0.05 mol/(g-enzyme), pH 7.80) under reduced pressure for 24 h.

2.3. Polymer–enzyme complex-catalyzed transesterification of Ac-L-PheOEt and 1-propanol in anhydrous isooctane

Ac-L-PheOEt and 1-propanol were dissolved in 20 ml of isooctane and the solution was used as a substrate solution for transesterification. The enzymatic transesterification of Ac-L-PheOEt with 1-propanol was started by adding the polymer–enzyme complex to the substrate solution. The respective initial concentrations of Ac-L-PheOEt, 1-propanol and polymer–enzyme complex were 5 mM, 500 mM, 0.01–0.5 g/l (native enzyme base). Isooctane was dried with a molecular sieve for 24 h prior to use. The water content was 50 mg/l, which was determined by Karl Fisher potentiometric titration using a moisture meter (AQ-7, Hiranuma, Japan). A control experiment was conducted with the same enzyme content of the lyophilized α-chymotrypsin, which was suspended in the reaction media by an ultrasound prior to the experiments. The temperature was kept at 40 °C. The reaction mixture was continuously agitated, and 3 μl of samples in the reaction mixture was periodically withdrawn after transesterification started. The concentration of Ac-L-PheOEt, Ac-L-PheOProp and Ac-L-Phe were quantitatively determined by high performance liquid chromatography (HPLC) which consisted of a UV8020 detector and a CCPS solvent delivery system (Tosoh, Japan), equipped with a Tosoh column ODS-80TS (4.6 i.d., 250 mm) using an acetonitrile/water (50/50, v/v) mixture adjusted to pH 7.00 containing 0.3% phosphoric acid as elution buffers with a flow rate of 1 ml/min (258 nm).

2.4. Kinetic analysis

Initial reaction rates for the transesterification of Ac-L-PheOEt and 1-propanol were determined from straight-line fits of the average value of the Ac-L-PheOProp formation based on HPLC measurements in triplicate. The apparent kinetic parameters were obtained by fitting the initial reaction data to the Michaelis–Menten equation (using KaleidaGraph). The percentage of active sites was determined for the various polymer–enzyme complexes in organic solvents, by observing spectrophotometrically the depletion of *N-trans*-cinnamoylimidazole in its unimolecular reaction with the enzyme preparations to form a relatively stable acyl-enzyme compound [25]. A two-fold molar excess (to enzyme) of *N-trans*-cinnamoylimidazole was dissolved in isooctane, and the solution was incubated at 25 °C for 90 min with mild shaking at 50 rpm. The polymer–enzyme complex was precipitated from the reaction mixture by adding 1 vol.% methanol, and solids were centrifuged. The supernatant containing unreacted *N-trans*-cinnamoylimidazole was analyzed using UV1200 spectrophotometer (Shimazu, Japan). The apparent value of k_{cat} was obtained by normalizing apparent V_{max} by the concentration of active enzyme determined in active site titration measurements.

3. Results and discussion

3.1. The effect of the enzyme concentration in the preparation stage on the activity of the polymer–enzyme complex

The concentration of enzyme in an aqueous solution from which the enzyme is recovered prior to use is sometimes a key parameter to govern the catalytic activity of noncovalently-formed enzyme preparations in organic media [6,13]. When preparing the noncovalently-formed surfactant–enzyme complex utilizing heterogeneous W/O emulsion, several thousands of enzyme molecules are entrapped in one emulsion droplet, thereby their activity decreases, because enzymes entrapped in emulsion droplets have no access to the substrates [13]. Fig. 1 shows the effect of enzyme concentration in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (for which molar ratio of the polymer/enzyme is 8) prepared from a succinic acid–sodium tetraborate buffer solution. The activity of PEG4000–α-chymotrypsin complex increased as the enzyme concentration increased, reached the maximal activity, and then gradually decreased. Although this is the same phenomenon which was observed in the preparation stage for the surfactant–enzyme complex [6,13], the reason seems to be different. The key factor for preparing the polymer–enzyme complex is to form a noncovalently-formed interaction between a polymer and an enzyme. The excess amount of polymers coated an active enzyme when the enzyme concentration is low, while the enzymes are aggregated and autodegraded due to a protein–protein interaction when the enzyme concentration is high, presumably resulting in the above phenomena. The optimal enzyme concentration for preparing the polymer–enzyme complex was therefore found to be ca. 0.5 g/l.

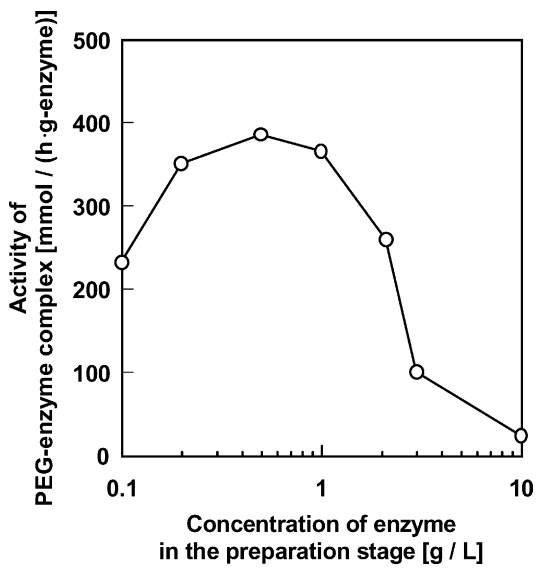

Fig. 1. The effect of the enzyme concentration in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (for which molar ratio of the polymer/enzyme was 8) prepared from a succinic acid–sodium tetraborate buffer solution (0.05 mol/(g-enzyme)) at pH 7.80.

3.2. The effect of the molar ratio of the polymer/enzyme in the preparation stage on the kinetic properties of the polymer–enzyme complex

One of the most important steps in the characterization of enzymes is to determine their kinetic properties. The determination of rate constants for enzymatic reactions provides valuable information in the detailed analysis and comparison of their reaction mechanisms. We previously found that the higher the molar ratio of the polymer/enzyme, the higher the activity of the polymer–enzyme complex obtained. The activity of the polymer–enzyme complex, however, gradually decreased with the increase in the molar ratio of the polymer/enzyme after reaching its maximum activity [21]. In this article, we kinetically evaluated these phenomena. To obtain kinetic properties of enzyme preparations in organic media, one must determine the percentage of active enzyme. A recent work, however, has shown that the methods for titration of active sites of enzyme preparations in organic media were erratic within ±10% [26]. Although we performed the active site titration for the PEG–α-chymotrypsin complex using *N-trans*-cinnamoylimidazole, erratic results were obtained within ±15%. This is more erratic than those reported for lyophilized subtilisin and α-chymotrypsin [26] presumably because of the molecular distribution of polymers used as modifier for the complex. The percentage of active sites ranged from a low of 19 ± 8% for PEG70000–α-chymotrypsin (100:1) complex to a high of 62 ± 15% for PEG4000–α-chymotrypsin (8:1) complex. Fig. 2 shows the effect of the molar ratio of the polymer/enzyme in the preparation stage

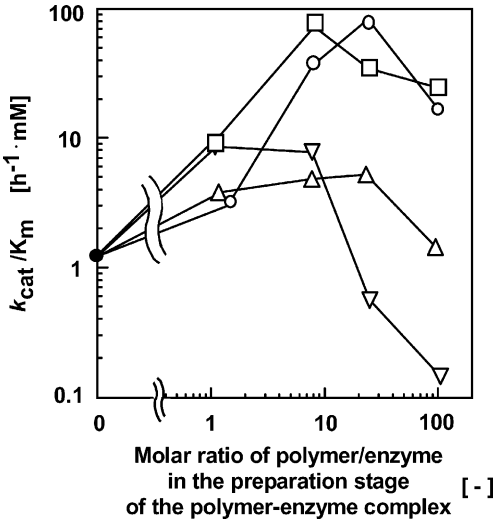

Fig. 2. The effect of the molar ratio of the polymer/enzyme in the preparation stage on the apparent kinetic properties of the PEG–α-chymotrypsin complex prepared from a KH_2PO_4–K_2HPO_4 buffer solution (0.05 mol/(g-enzyme)) at pH 7.80 (closed circle: native α-chymotrypsin, open circle: PEG1000–α-chymotrypsin complex, open square: PEG4000–α-chymotrypsin complex, open triangle: PEG20000–α-chymotrypsin complex, open reverse triangle: PEG70000–α-chymotrypsin complex). The concentration of enzyme in the preparation stage was 0.5 g/l.

on k_{cat}/K_m of PEG–α-chymotrypsin complexes prepared from a KH_2PO_4–K_2HPO_4 buffer solution. The apparent turnover number, k_{cat}, increased as the molar ratio of the polymer/enzyme increased, reached the maximal value, and then gradually decreased. On the contrary, the apparent Michaelis constant, K_m, decreased as the molar ratio of the polymer/enzyme increased, reached the minimal value, and then gradually increased (data not shown). These phenomena correspond to the change in the favorable microenvironment created around the surface of the enzyme molecule by the hydrophilic head group of PEG, as reported for the enzyme preparations using polymers such as palmitoyl poly(sucrose acryloate)–subtilitin Carlsberg complex [16] and lipase chemically modified with PEG [27], thereby the polymer–enzyme complex showed a high catalytic activity in organic media. As a result, the k_{cat}/K_m increased as the molar ratio of the polymer/enzyme increased, reached the maximal value, and then gradually decreased, as shown in Fig. 2. The optimal molar ratio of the polymer/enzyme in the preparation stage was found to be ca. 8.

Additionally, recent reports have shown that solubilized enzymes showed a high catalytic activity in organic media. The ion-paired surfactant–α-chymotrypsin complex that showed an aqueous-like activity was soluble in isooctane at least at 1 g/l [28]. The surfactant–enzyme complex was soluble in benzene at 1 g/l [9] and toluene at 0.4 g/l [29] while insoluble in ethanol, chloroform and dichloroethane [29]. On the other hand, subtilisin chemically modified with poly(ethylene glycol) monomethacrylate was soluble in a variety of organic solvents up to 5 g/l [30]. In our case, solubility measurements at 40 °C showed that the roughly-estimated solubility of the PEG–α-chymotrypsin complex (with a molar ratio of the polymer/enzyme of 8) prepared from a KH_2PO_4–K_2HPO_4 buffer solution was 0.4 g/l in ethanol, 0.7 g/l in isooctane, 0.9 g/l in chloroform and over 3 g/l in benzene. These results suggested that the polymer–enzyme complex was a good soluble biocatalyst for nonaqueous enzymology, and that the solubilization of modified enzymes in organic solvents was crucial for their activation.

3.3. The effect of the pH of buffer solutions in the preparation stage on the activity of the polymer–enzyme complex: does the polymer–enzyme complex demonstrate "pH memory" effect?

Enzymes demonstrate a "pH memory" effect, i.e. the ionization state of an enzyme in organic media remains the same as in the last aqueous solution from which the enzyme is recovered [31,32]. Fig. 3 shows the effect of the pH of buffer solutions in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (with a molar ratio of the polymer/enzyme of 8) prepared from a boric acid–NaOH, a boric acid–sodium tetraborate and a succinic acid–sodium tetraborate buffer solutions. The pH-dependence of the activity of the polymer–enzyme complex in anhydrous isooctane was strongly influenced by the pH of aqueous buffer solutions from which the complex is recovered. The optimal pH for the activity of the polymer–enzyme complex was found to be ca. pH 7.5–8.0, which is almost the same as that for the activity of native α-chymotrypsin in an aqueous solution, ca. 7.80 (data not shown). This result strongly suggested that the polymer–enzyme complex demonstrated the "pH memory" effect and

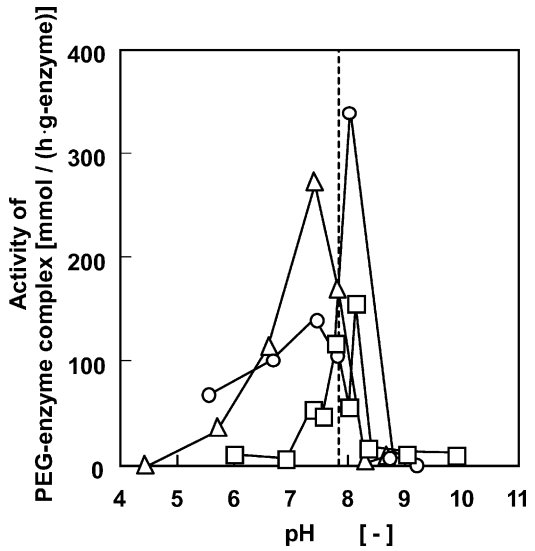

Fig. 3. The effect of the pH of buffer solutions in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (for which molar ratio of the polymer/enzyme was 8) prepared from boric acid–NaOH (open square), boric acid–sodium tetraborate (open circle) and succinic acid–sodium tetraborate (open triangle) buffer solutions (0.05 mol/(g-enzyme)). The concentration of enzyme in the preparation stage was 2.11 g/l. The dotted line represents the optimal pH for the transesterification of Ac-L-PheOEt and 1-propanol in an aqueous solution, i.e. 7.80.

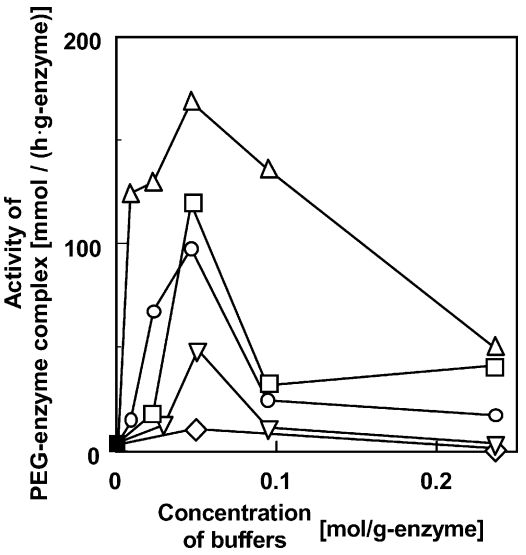

Fig. 4. The effect of the buffer concentration (pH 7.80) in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (for which molar ratio of the polymer/enzyme was 8). The buffers used were boric acid–NaOH (open square), boric acid–sodium tetraborate (open circle), succinic acid–sodium tetraborate (open triangle), succinic acid–NaOH (open reverse triangle) and potassium dihydrogenphosphate–sodium tetraborate (open lozenge). The concentration of enzyme in the preparation stage was 2.11 g/l. Closed square represents the result for the polymer–enzyme complex using no buffer agents.

the pH adjustment at its preparation stage was crucial for its activation in organic media.

3.4. The effect of the buffer concentration in the preparation stage on the activity of the polymer–enzyme complex

The effect of the buffer concentration in the preparation stage on the activity of the polymer–enzyme complex was investigated using 16 buffers. Fig. 4 shows the effect of the buffer concentration (adjusted at pH 7.80) in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (with a molar ratio of the polymer/enzyme of 8) prepared from five buffer (a boric acid–NaOH, a boric acid–sodium tetraborate, a succinic acid–NaOH, a succinic acid–sodium tetraborate and a potassium dihydrogenphosphate-sodium tetraborate) solutions. The buffer may protect the enzyme from direct inactivation by the organic solvent, or help to maintain the native conformation of the enzyme during lyophilization. The activity of the polymer–enzyme complex thus increased as the buffer concentration increased up to 0.05 mol/(g-native enzyme). This is a common phenomenon for enzyme preparations in organic media, as reported for a polymer-coated enzyme [17] and native enzyme [33] We found, however, the activity of the polymer–enzyme complex, finally, decreased gradually after reaching its maximal value as shown in Fig. 4. This result suggested that the excess adsorption of buffer on the enzyme surface prevented the effective contact between the enzyme active site and the substrates. The optimal buffer concentration in the preparation stage was found to be ca. 0.05 mol/(g-enzyme) for all enzyme preparations.

3.5. The effect of buffer nature in the preparation stage on the activity of the polymer–enzyme complex: a succinic acid–sodium tetraborate buffer dramatically enhanced the activity of the complex

Fig. 5 shows the effect of buffer nature (adjusted at pH 7.80, 0.05 mol/(g-enzyme) in the preparation

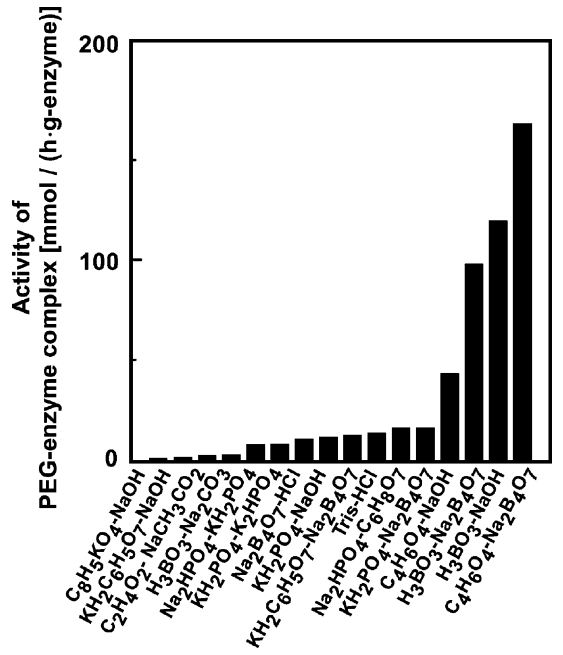

Fig. 5. The effect of the buffer nature (pH 7.80) in the preparation stage on the activity of the PEG4000–α-chymotrypsin complex (for which molar ratio of the polymer/enzyme was 8). The concentration of enzyme in the preparation stage was 2.11 g/l. The buffer concentration was 0.05 mol/(g-enzyme) in all cases.

stage prior to lyophilization on the activity of the PEG4000–α-chymotrypsin complex (with a molar ratio of the polymer/enzyme of 8) in anhydrous isooctane. We recently reported that a boric acid–NaOH buffer was useful for the activation of the complex, and succeeded in preparing the poly(ethylene glycol)–α-chymotrypsin complex which showed ca. 6000-fold higher activity than native α-chymotrypsin in anhydrous isooctane [22]. In this article, however, we found that the activity of the polymer–enzyme complex was greatly dependent on the buffer property, i.e. a succinic acid–sodium tetraborate buffer dramatically activated the polymer–enzyme complex, while a potassium hydrogenphthalate–NaOH buffer almost deactivated the complex. Skrika-Alexopoulos and Freedman reported the same kind of buffer effects, i.e. bilirubin oxidase lyophilized from AMPSO buffer solution was extremely active, while it was completely deactivated using a volatile buffer solution [33]. The information on the effect of buffers present during lyophilization on the activity of α-chymotrypsin preparations in organic media has been limited to date. Phosphates are commonly-used buffers for α-chymotrypsin preparations in organic media, such as surfactant–α-chymotrypsin complex [6] and salt-activated α-chymotrypsin [23,24], while sodium tetraborate was generally used for α-chymotrypsin chemically modified with PEG [34]. Results in Fig. 5 clearly showed that a series of buffers containing boric acid, sodium tetraborate and succinic acid was effective to activate the polymer–enzyme complex in anhydrous isooctane. For comparison, we tested the buffer effect on the activity of α-chymotrypsin in an aqueous solution using some transesterification, hydrolysis and synthetic reactions (data not shown). Interestingly, a borate buffer showed no effect on the enzyme activation. For example, the respective activity of native α-chymotrypsin was 3.78, 5.20 and 4.12 mmol(h (g-enzyme)) in a succinic acid–sodium tetraborate, a potassium hydrogenphthalate-NaOH and Tris–HCl buffer solutions (pH 7.80, 0.05 mol/(g-enzyme)) using α-chymotrypsin-catalyzed hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide as a model reaction. These results suggested that the dramatic activation of the polymer–enzyme complex induced by a succinic acid–sodium tetraborate buffer did not result from the activation effect in the preparation stage prior to lyophilization.

Ru et al. recently reported interesting phenomena that the co-lyophilization with simple inorganic salts such as KCl at a very high concentration (>90 wt.%) enhanced the activity of native enzymes in organic media, and the phenomenon was governed by the Jones–Dole B coefficient, a thermodynamic parameter for characterizing the salt's affinity for water and its chaotropic or kosmotropic character [23,24]. The group also reported that dramatic activation (ca. 50-fold) of α-chymotrypsin was observed at a very high concentration of inorganic salts (94 wt.%, i.e. 0.252 mol/(g-enzyme)) [35]. Their reports on the matrix formation between enzymes and an inorganic salt at a very high concentration were of great interest for the dramatic activation of enzymes in organic media. In our experiments, we tested the effect of buffer agents at a low concentration (0.05 mol/(g-enzyme)) (without inorganic salts) on the activity of polymer–enzyme complex in organic media. The degree of activation induced by a succinic acid–sodium tetraborate buffer (over a potassium

dihydrogenphosphate–dipotassium hydrogenphosphate buffer) was estimated to be ca. 23 as shown in Fig. 5, which was of the same order as that reported by Ru et al. (i.e. 50) who used inorganic salts at a very high concentration as activator. These results suggested the proper choice of buffer agents was effective for the enzyme activation in organic media.

The rational reason for the phenomena obtained in this article (i.e. the great dependence of the activity of the complex on the buffer concentration) is still unclear now; however, some possible explanations can be shown. The different buffer agents might differ in their interaction with residual water (the activity of enzyme preparations was greatly dependent on the residual water content in the enzyme matrix as reported by Ru et al. [23]). On the other hand, the different buffer agents may differ in their ability to interact with an enzyme and substrates. Also, there is a possibility that the different buffer agents differ in their ability of stabilizing enzymes, like salt effects as frequently reported for protein chemistry in aqueous solution [36]. Further work concerning the buffer effect on the activity of enzyme preparations in organic media is required to establish their optimal design methodology for nonaqueous enzymology.

3.6. The effect of the polymer nature in the preparation stage on the activity of the polymer–enzyme complex: only amphiphilic polymers gave the activated complex

The effect of the polymer nature on the activity of the polymer–enzyme complex in organic media was tested using 10 polymers. We classified these polymers into three groups: relatively-hydrophobic (polystyrene, poly(vinyl formal) and poly(vinyl chloride)), relatively-hydrophilic (poly(vinyl alcohol), poly(tetramethylene oxide), ethyl cellulose, dextran, and poly(vinyl butyral)) and amphiphilic groups (PEG4000 and poly(vinyl pyrrolidone)). The classification only depended on the preliminary solubility experiments in water, isooctane and chloroform (which was sometimes used as a polymer-dissolving medium [18]) at 40 °C (data not shown). Fig. 6 shows the effect of polymer nature on the activity of the polymer–enzyme complex in anhydrous isooctane. A succinic acid–sodium tetraborate buffer was used (pH 7.80, 0.05 mol/(g-native enzyme)) prior to lyophilization in all cases. We prepared each complex under the condition that the weight ratio (not molar ratio as used in other experiments in this article) of polymer/enzyme was unity, because average molecular weights of some polymers were unknown from vendor. This treatment did not affect the subsequent discussion, because in various weight ratio conditions, hydrophobic and hydrophilic polymers had no dramatic effect on enzyme activation (i.e. the activity of the complex using hydrophobic and hydrophilic polymers was always low).

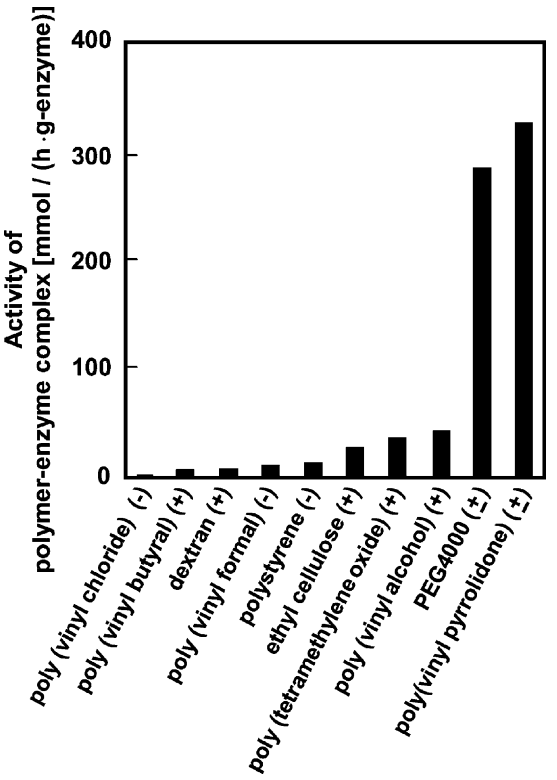

Fig. 6. The effect of the polymer nature on the activity of the polymer–enzyme complex in anhydrous isooctane (plus: hydrophilic polymers, minus: hydrophobic polymers and plus-minus: amphiphilic polymers). A succinic acid–sodium tetraborate buffer was used (pH 7.80, 0.05 mol/(g-enzyme)) prior to lyophilization in all cases. The weight ratio of polymer/enzyme was unity. The concentration of enzyme in the preparation stage was 2 g/l.

We found only amphiphilic polymers (PEG4000 and poly(vinyl pyrrolidone)) activated the polymer–enzyme complex in anhydrous isooctane, as clearly shown in Fig. 6. It was difficult to prepare the

complex formed with α-chymotrypsin and hydrophobic polymers, i.e. polymers suspended in an aqueous buffer solution in the preparation stage due to their hydrophobicity, while we obtained homogeneous white powder when hydrophilic and amphiphilic polymers were used as modifiers. This suggested that a hydrophilic nature of hydrophilic and amphiphilic polymers facilitated their complexation with an enzyme, while hydrophobic polymers are not useful for forming the complex. On the other hand, the complex prepared from hydrophilic polymers was completely suspended in an organic solvent. These results strongly suggested that amphiphilic nature of polymers was important to prepare the polymer–enzyme complex in an aqueous solution, and subsequently used in organic media. We finally succeeded in preparing the poly(vinyl pyrrolidone)–α-chymotrypsin complex which showed ca. 15,000-fold higher activity than native α-chymotrypsin in anhydrous isooctane under the same preparation conditions (Fig. 6).

4. Conclusions

Although our model enzyme, α-chymotrypsin, is one of the most extensively studied enzyme with known three-dimensional structure and catalytic mechanism, it is difficult to activate it in anhydrous organic media. Some groups have reported that chemical and physical modifications are effective to prepare highly-active modified α-chymotrypsin in organic solvents. We demonstrated here that the novel simple physical modification was effective to dramatically activate α-chymotrypsin in organic solvents. Optimal preparation conditions were obtained when poly(ethylene glycol)4000 was used as a modifier of α-chymotrypsin for the transesterification of Ac-L-PheOEt and 1-propanol in anhydrous isooctane. Additionally, we found that the amphiphilic nature of polymers was important to prepare the polymer–enzyme complex in an aqueous buffer solution, and subsequently used in an organic media. We finally succeeded in preparing the poly(vinyl pyrrolidone)–α-chymotrypsin complex which showed ca. 15,000-fold higher activity than native α-chymotrypsin in anhydrous isooctane under the same preparation conditions. Our modification method can be applied to other enzymes such as thermolysin and lipase (data not shown). We thus believe the optimization methodology in this article can be very attractive for nonaqueous enzymology.

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