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Influence of Water Activity on Protease Adsorbed on Biochar in Organic Solvents

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Abstract

We have found that the organic solvent-resistance of α-chymotrypsin (α-CT) is enhanced by adsorbing α-CT onto bamboo charcoal powder (BCP), which is obtained by pyrolyzing bamboo waste under nitrogen atmosphere, and is markedly dependent on the thermodynamic water activity (a_w) in organic solvents. When BCP-adsorbed α-CT was immersed in acetonitrile at an appropriate water activity, it effectively enhanced the transesterification of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with *n*-butanol (BuOH) to produce *N*-acetyl-L-tyrosine butyl ester (*N*-Ac-Tyr-OBu), compared to the hydrolysis of *N*-Ac-Tyr-OEt with water to give *N*-acetyl-L-tyrosine (*N*-Ac-Tyr-OH). When the water activity was 0.28, the initial rate of transesterification catalyzed by BCP-adsorbed α-CT was about sixty times greater than that catalyzed by free α-CT. Regarding the reaction selectivity which is defined as a ratio of the initial rate of transesterification to that of hydrolysis, BCP-adsorbed α-CT was much superior to free α-CT. The catalytic activity of BCP-adsorbed α-CT was markedly dependent on the reaction temperature. Furthermore, concerning the thermal stability at 50 °C, the half-life of BCP-adsorbed α-CT exhibited 3.8-fold, compared to that of free α-CT.

Keywords: adsorption, α-chymotrypsin, biochar, enzymatic activity, thermal stability, water activity

1. Introduction

The utilization of biomass wastes, which are carbon neutral, for energies and functional materials is one of the most important challenges to reduce greenhouse gas emissions (Ho & Show, 2015; Straathof, 2014). However, almost all forestry residues have hardly been used. Accordingly, the development in the high value-added application of forestry residues has been desired to provide the multiple effective utilization system of forestry residues.

On the other hand, environmentally benign processes such as biotransformation, biosensor, biofuel cell, and so on have recently been developed by using enzymes as a kind of proteins, since enzymes exhibit their outstanding biological activity under mild conditions (Buchholz et al., 2012; Silwana et al., 2014; Leech et al., 2012). However, enzymes are gradually denatured and inactivated under various physical and chemical stresses such as heat, organic solvents, and so on, although they are generally stable in a cell (Bailey & Ollis, 1986). To enhance the stress resistance of enzymes, enzymes have been modified chemically and genetically (Buchholz et al., 2012; Cioci & Lavecchia, 1998). As a chemical modification, enzyme immobilization and stabilizers have been developed, and molecular engineering has been carried out as a genetic modification. Among these enzyme modifications, an enzyme immobilization system has widely been studied due to following advantages (Buchholz et al., 2012): 1. Concentration of substrate can be increased. 2. Recycled enzymes can be used many times. 3. Separation of the products is straight forward. 4. Stability of the enzyme to change under stress is increased. Especially, the adsorption of enzymes onto various carriers has been widely used from the laboratory scale to the industrial scale because of the simplest and most economical method of stabilizing enzymes (Elnashar, 2010; Mateo et al., 2007). The physical and chemical surface properties of carriers strongly affect the performances of adsorbed enzymes such as the catalytic activity, the specificity, and the stability. Thus, it is probably that the stress resistance of enzymes is enhanced by selecting an appropriate carrier.

As a part of our ongoing research efforts aimed at utilizing forestry residues, we have so far examined the usefulness of biomass charcoal powder, which is prepared from forestry residues by pyrolysis, as an enzyme carrier. We have found that enzymes are effectively adsorbed onto biomass charcoal powder (Noritomi et al., 2013a; Noritomi et al., 2013b), and biomass charcoal powder imparts high heat stress resistance to enzymes through the adsorption (Noritomi et al., 2011; Noritomi et al., 2012; Noritomi et al., 2014; Noritomi et al., 2016). Furthermore, we have reported that the adsorption of enzymes onto biomass charcoal powder can sufficiently improve the organic solvent resistance of enzymes in hydrophilic organic solvents (Noritomi et al., 2017). When solid enzymes are at moist air, the catalytic activity of enzymes strongly depends on the thermodynamic water activity (a_w), which is the ratio of water partial pressure to vapor pressure of pure water (Acker, 1962). Likewise, since adsorbed enzymes are solids in hydrophilic organic solvents containing low water content, it is probably that the catalytic activity of adsorbed enzymes is influenced by the water activity. However, there have been few reports regarding the relation between the water activity in hydrophilic organic solvents and the performance of adsorbed enzymes.

In our present work, we have assessed how the water activity affects the organic solvent resistance of enzymes adsorbed onto biomass charcoal powder in hydrophilic organic solvents. We have used bovine pancreas α-chymotrypsin (α-CT) as a model enzyme since it is well investigated regarding its structure, functions, and properties (Kumar & Venkatesu, 2012).

2. Method

2.1 Materials

α-Chymotrypsin (EC 3.4.21.1 from bovine pancreas) (type II, 52 units/mg solid) (α-CT) was purchased from Sigma-Aldrich Co. (St. Louis, USA). *N*-Acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) and *N*-acetyl-L-tyrosine (*N*-Ac-Tyr-OH) were also from Sigma-Aldrich Co. (St. Louis, USA). Acetonitrile of guaranteed grade was obtained from Kanto Chemical Co. (Tokyo, Japan). Before acetonitrile was used as a reaction solvent, it was dried by storing it over dry 0.3 nm molecular sieves (Wako Chemical Co.) for at least 24 h.

2.2 Preparation of Bamboo Charcoal Powder

To prepare bamboo charcoal, under nitrogen atmosphere, bamboo waste was dried at 180 ℃ for 2 hr, was pyrolyzed at 450 ℃ for 2 hr, was carbonized at 350 ℃ for 3 hr, and then was cooled at 100 ℃ for 1 hr by pyrolyzer (EE21 Pyrolyzer, EEN Co. Ltd., Japan). Bamboo charcoal powder (BCP) was obtained by grinding the resultant bamboo charcoal with jet mill (100AS, Fuji Sangyo Co. Ltd., Japan).

2.3 Characterization of Bamboo Charcoal Powder

The SEM micrograph was obtained using a scanning electron microscope (JSM-7500FA, JEOL, Japan) operating at 15 kV. The sample for SEM was prepared on a carbon tape without vapor deposition.

All samples were outgassed at 300°C for 8 h prior to the nitrogen adsorption measurements. The specific surface area of BCP was calculated with the use of the Brunauer-Emmett-Teller (BET) method using a micropore system (BELSORP-mini II, BEL JAPAN, INC.).

The surface of BCP was analyzed by X-ray photoelectron spectroscopy (XPS) (Quantum-2000, ULVAC-PHI Co. Ltd.) operating at an x-ray beam size of 100 μm.

2.4 Adsorption of α-Chymotrypsin onto Bamboo Charcoal Powder

As a typical procedure, 5 mL of 0.01 M phosphate buffer solution at pH 7 containing 300 μM α-CT and 3 g/L BCP was placed in a 10-mL test tube with a screw cup, and was incubated at 25 ℃ and 120 rpm for 24 h. After adsorption, the mixture was filtrated with a membrane filter (pore size: 0.1 μm, Millipore Co. Ltd.), collected BCP-adsorbed α-CT was rinsed by dry acetonitrile several times, and was dried sufficiently. The amount of α-CT adsorbed onto BCP was calculated by subtracting the amount of α-CT included in the supernatant liquid after adsorption from the amount of α-CT in the aqueous solution before adsorption. The amount of α-CT was measured at 280 nm by UV/vis spectrophotometer (UV-1800, Shimadzu Co. Ltd.).

2.5 Measurement of Catalytic Activity of α-Chymotrypsin

The standard reaction for transesterification was carried out as follows: Three milliliter of acetonitrile containing a certain amount of water, 10 mM *N*-Ac-Tyr-OEt, 1000 mM *n*-butanol, 1 mM acetanilide, and free α-CT or BCP-adsorbed α-CT (30 μM) was placed in a 4-mL screw-cap vial, and was incubated at 120 rpm and 25 °C. The amounts of the reaction components were periodically determined with HPLC (Shimadzu LC-10A) (Shimadzu Co., Kyoto, Japan) using a TSK-GEL ODS-80TM column (Tosoh Co., Tokyo, Japan) eluted with water-acetonitrile (6:4 by volume) at 0.5 mL/min with detection at 270 nm. Acetanilide was used as an internal standard.

2.6 Measurement of Remaining Activity of α-Chymotrypsin

In order to assess the thermal stability of free α-CT and BCP-adsorbed α-CT, the catalytic activity of free α-CT or BCP-adsorbed α-CT was measured at the water activity of 0.15 after free α-CT or adsorbed α-CT was stored in acetonitrile at 50 ℃ for appropriate time, and then was cooled at 25 ℃ for 30 min. The remaining activity was obtained by Equation (1).

$$\text{Remaining activity (\%)} = \frac{\text{Catatytic activity after heat treatment}}{\text{Catalytic activity before heat treatment}} \times 100 \quad (1)$$

2.7 Measurement of Fourier Transform Infrared (FTIR) Spectroscopy

FTIR measurements of free α-CT and BCP-adsorbed α-CT were carried out using a Jasco FT/IR spectrometer model FT/IR-4100. A KBr pellet containing 0.5 mg of free α-CT or BCP-adsorbed α-CT powder per 100 mg of KBr was prepared, and the measurements were performed using 512 scans under 4.0 cm^{-1} resolution.

3. Results and Discussion

3.1 Characterization of Bamboo Charcoal Powder

We have pyrolyzed bamboo waste at low temperatures under nitrogen atmosphere to produce functional groups, which were used as a binding site for the adsorption of enzymes. The fine bamboo charcoal powder was obtained by grinding the resultant bamboo charcoal with jet mill. The mean diameter of BCP was 7 μm.

Table 1 shows the characteristics of BCP. The specific surface area of BCP was one order of magnitude lower than that of activated carbon, which is used as an adsorbent, and was the pore volume of BCP was low as well. Moreover, the pore diameter peak was much smaller than the size of α-CT since the size of α-CT is 5.1 x 4.0 x 4.0 nm (Kumar & Venkatesu, 2012).

Table 1. Characteristics of BCP

Textural property	Experimental value
Specific surface area (m^2/g)	294
Pore volume (cm^3/g)	0.041
Pore diameter peak (nm)	Less than 2.6

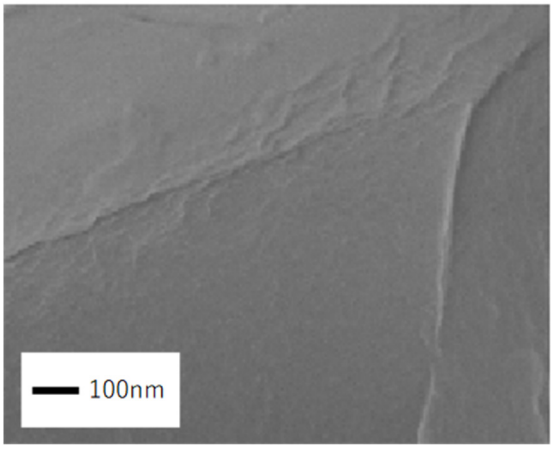

Figure 1. SEM images of bamboo charcoal powder (BCP)

Figure 1 shows the scanning electron micrograph of BCP. The surface of BCP was almost smooth. It is probably that micropores were not observed under the magnification measured in the present work since the pore size was several nanometers or less.

To estimate the chemical property of the surface of BCP, BCP has been measured by X-ray photoelectron spectroscopy (XPS). As shown in Table 2, carbon atom was the main element, oxygen atom was also located on the surface of BCP to some extent, and nitrogen atom was detected at a small ratio. Furthermore, by the measurement of narrow scan spectra of XPS, C-C, C-H, C-O, O-C-O, C=O, COOH, and C-N were detected as a chemical state of carbon.

BCP-adsorbed α-CT was obtained by dispersing BCP in the buffer solution at pH 7 containing α-CT, and the amount of α-CT adsorbed onto BCP was 9.8 μmol/g. The charge of α-CT is positive in the buffer solution at pH 7 since the isoelectric point of α-CT is 9.1 (Kumar & Venkatesu, 2012). On the other hand, the ζ-potential of BCP is negative at pH 7 in the buffer solution at pH 7 (Noritomi et al., 2013[a]). Accordingly, it is suggested that the adsorption is mainly due to the electrostatic attraction between the positively charged α-CT and the negatively charged surface of bamboo charcoal powder.

Table 2. Elemental ratio of BCP measured by X-ray photoelectron spectroscopy

Element	Atomic percentage (%)
Carbon	80.7
Nitrogen	0.8
Oxygen	15.9
Other	2.6

3.2 Dependence of Catalytic Activity of BCP-Adsorbed α-CT on Water Activity in Acetonitrile

An enzymatic reaction in hydrophilic solvents has the advantage of the solubility of a variety of substrates, including amino acid derivatives, which are poorly soluble in hydrophobic solvents (Kise et al., 1990). However, when a hydrophilic solvent is used as a reaction medium, the enzyme molecule directly comes in contact with the solvent, and thereby its catalytic activity is strongly influenced by the nature of the solvent (Klibanov, 2001; Noritomi et al., 2007). Moreover, the catalytic activity of enzymes tends to be reduced since hydrophilic solvents tear water from enzymes. Water bound to enzymes is essential for enzymes to maintain their mobility for catalysis (Klibanov, 1997). Accordingly, it is considered that the performance of enzymes is strongly influenced by the water activity in hydrophilic solvents. To assess the effect of water activity on the catalytic activity of BCP-adsorbed α-CT in acetonitrile, we have examined the relationship of initial rates of transesterification and hydrolysis with the water activity in acetonitrile. The water activity was prepared by adding an appropriate amount of water to acetonitrile (Bell et al., 1997). Figure 2 shows the plots of the initial rate (V_e) of transesterification and the initial rate (V_h) of hydrolysis against the water activity in acetonitrile at 25 °C. The inherent enzymatic hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with water was inhibited by low water activity, while the enzymatic transesterification of *N*-acetyl-L-tyrosine ethyl ester (*N*-Ac-Tyr-OEt) with *n*-butanol (BuOH) was promoted. The initial rates of transesterification catalyzed by BCP-adsorbed α-CT and free α-CT were strongly dependent on the water activity, and displayed a bell-shaped curve. The initial rate of transesterification catalyzed by BCP-adsorbed α-CT was about sixty times greater than that catalyzed by free α-CT when the water activity was 0.28, at which the maximum initial rates of transesterification of free α-CT and BCP-adsorbed α-CT were obtained. On the other hand, the initial rates of hydrolysis catalyzed by free α-CT and BCP-adsorbed α-CT increased with an increase in the water activity. The relationship between the catalytic activity of enzymes and the water activity in organic solvents tends to exhibit bell-shaped curve. The optimal water activity results from the balance between the kinetic rigidity of enzyme structures and their thermodynamic stability (Klibanov, 2001; Bell et al., 1997). The kinetic rigidity decreases with increasing water activity, while the native enzyme structure gradually changes through thermodynamic stability. Accordingly, the catalytic activity of enzymes increases with an increase in the flexibility of rigid enzyme structures, and then decreases with an increase in the disturbance of enzyme structures. On the other hand, since the increase of water activity results in the increase of overall water concentration, the hydrolysis is enhanced by the increase of water activity. Regarding the reaction selectivity, which was the ratio of the initial rate (V_e) of transesterification to the initial rate (V_h) of hydrolysis, BCP-adsorbed α-CT was much superior to free α-CT.

Table 3 shows the ratio of the absorbance at 1650 cm^{-1} to the absorbance at 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of BCP-adsorbed α-CT. The bands at ca. 1650 and 1630 cm^{-1} are assignable to α-helix and intramolecular β-sheet, respectively (Surewicz & Mantsch, 1988). The higher absorbance ratio, the higher secondary structure. Since the absorbance ratio (ABS_{1650}/ABS_{1630}) of BCP-adsorbed α-CT was independent on the water activity, the secondary structure of BCP- adsorbed α-CT was not influenced by the water activity. Thus, the secondary structure of BCP-adsorbed α-CT is firmly maintained through the adsorption. The absorbance ratio (ABS_{1650}/ABS_{1630}) of BCP-adsorbed α-CT is higher than that of free α-CT (Noritomi et al., 2017). The results indicate that the water activity effectively affects the catalytic activity of BCP- adsorbed α-CT having a high second structure, compared to that of free α-CT.

Table 3. Ratio of the absorbance at 1650 cm^{-1} to the absorbance at 1630 cm^{-1} (ABS_{1650}/ABS_{1630}) of BCP-adsorbed α-CT provided by the FTIR measurement

Water activity (-)	ABS_{1650}/ABS_{1630} (-)
0.03	1.3
0.28	1.3
0.55	1.3
0.73	1.3

3.3 Temperature Dependence of Catalytic Activity of BCP-Adsorbed α-CT in Acetonitrile

Figure 3 shows the plots of initial rate (V_e) of transesterification and the initial rate (V_h) of hydrolysis in acetonitrile against reaction temperature when the water activity was 0.55 at 25 °C. The initial rate of transesterification catalyzed by BCP- adsorbed α-CT exhibited a maximum around 35 °C, and then decreased with an increase in temperature, while that catalyzed by free α-CT slightly increased with increasing temperature. The initial rate of transesterification catalyzed by BCP- adsorbed α-CT exhibited 30-fold, compared to that catalyzed by free α-CT at 35 °C. On the other hand, the initial rate of hydrolysis catalyzed by BCP- adsorbed α-CT showed a maximum around 40 °C, and then decreased with an increase in temperature, while that catalyzed by free α-CT showed a slight increase with increasing temperature, as well as the case of transesterification. The initial rate of hydrolysis catalyzed by BCP- adsorbed α-CT was thirteen times greater than that catalyzed by free α-CT at 40 °C. Enzymatic reactions obey the Arrhenius correlation between the reaction rate constant and the reaction temperature as well as chemical reactions (Volkin & Klibanov, 1989). Thus, the initial rate becomes higher at higher temperatures. However, since the thermal denaturation of enzymes proceeds with increasing temperature due to the disruption of weak interactions in enzymes including ionic bonds, hydrogen bonds, and hydrophobic interactions, which are prime determinants of enzyme tertiary structures. On the other hand, the water activity is influenced by temperature (Reid et al., 1987). Consequently, it is probably that the optimal temperature of the catalytic activity of BCP-adsorbed α-CT was observed due to these factors. The result indicates that the catalytic activity of α-CT is sufficiently enhanced by adsorbing α-CT onto BCP since the initial rate of transesterification catalyzed by BCP-adsorbed α-CT was about fifteen times greater than that catalyzed by free α-CT even at 50 °C.

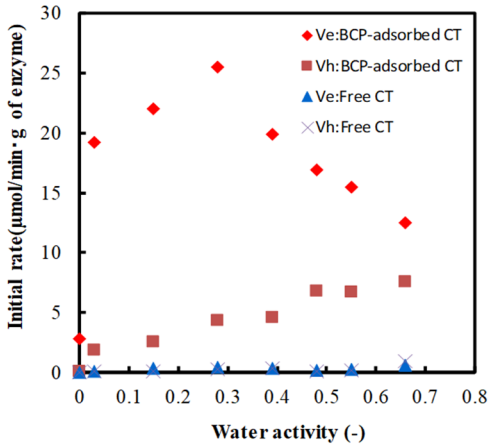

Figure 2. Effect of water activity on the initial rate (V_e) of transesterification and the initial rate (V_h) of hydrolysis in acetonitrile

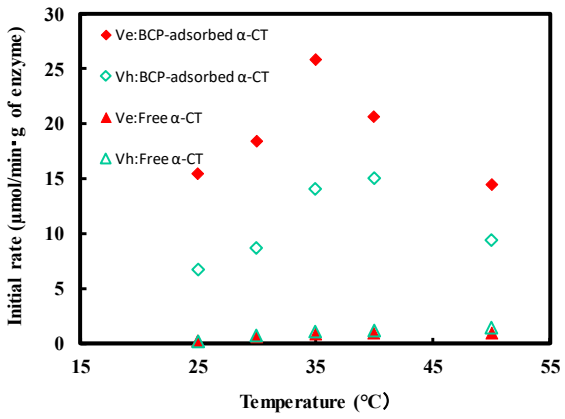

Figure 3. Temperature-dependence of the initial rate (V_e) of transesterification and the initial rate (V_h) of hydrolysis in acetonitrile

3.4 Thermal Stability of BCP- Adsorbed α-CT in Acetonitrile

Heating time directly enhances the denaturation of enzymes (Volkin & Klibanov, 1989). Figure 4 shows time course of remaining activities of free α-CT and BCP- adsorbed α-CT through the heat treatment at 50 °C when the water activity was 0.15 at 25 °C. Free α-CT in acetonitrile, where α-CT was dispersed as the solid state, was unchanged during the heat treatment, although enzymes dissolved in an aqueous solution immediately form the aggregation of thermally-denatured enzymes (Noritomi et al., 2011). Likewise, the enzyme aggregation and the cohesion among BCP- adsorbed α-CT were not observed in acetonitrile during the heat treatment. However, the

remaining activities of free α-CT and BCP-adsorbed α-CT gradually decreased with heat time. As seen in the figure, the relationship of the remaining activities of free α-CT and BCP- adsorbed α-CT with heat time could be correlated by first-order and second-order kinetics, respectively. The half-lives of inactivation of free α-CT and BCP- adsorbed α-CT calculated from the fitting curves in the figure were 33 and 125 min, respectively. Thus, the half-life of BCP- adsorbed α-CT exhibited 3.8-fold, compared to that of α-CT. On the other hand, we have reported that the half-lives of inactivation of BCP- adsorbed α-CT is 15 min in an aqueous solution at 45 ℃ (Noritomi et al., 2014). Thus, the thermal stability of BCP- adsorbed α-CT in acetonitrile was much superior to that of BCP-adsorbed α-CT in water. The result indicates that the adsorption is strengthened in acetonitrile by the electrostatic force, which mainly contributes to the adsorption of α-CT onto BCP, and stabilizes α-CT since the dielectric constant of acetonitrile is much lower than that of water (Reichardt, 1988).

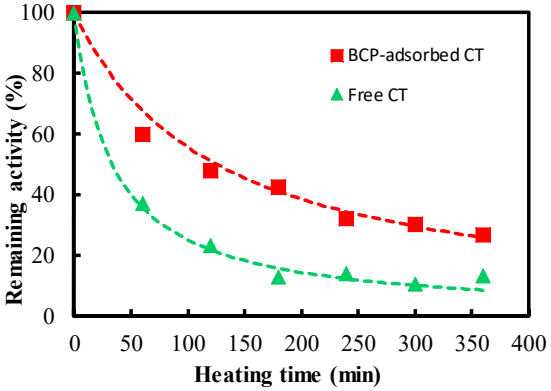

Figure 4. Time course of remaining activities of free α-CT and BCP-adsorbed α-CT through the heat treatment at 50 ℃

4. Conclusions

We have demonstrated that the water activity affects the catalytic activity of α-CT in acetonitrile. The catalytic activity of BCP-adsorbed α-CT was more effectively enhanced by changing the water activity than that of free α-CT. The catalytic activity of BCP-adsorbed α-CT was strongly influenced by reaction temperature. The catalytic activity of BCP-adsorbed α-CT exhibited the optimum temperature, and was much greater than that of free α-CT. The thermal stability of α-CT was markedly improved by adsorbing α-CT onto BCP, and the half-life of BCP-adsorbed α-CT exhibited 3.8-fold, compared to that of α-CT.

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