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Thermal Stabilization of HEWL by Adsorption on Biochar

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Abstract

We have found that the heat stress tolerance of hen egg white lysozyme (HEWL) is markedly enhanced by the adsorption of HEWL on bamboo charcoal powder (BCP), which is a kind of biochar. HEWL was firmly adsorbed on BCP even at high temperatures. The secondary structure of HEWL was altered to some extent by the adsorption of HEWL on BCP. The remaining activity of BCP-adsorbed HEWL exhibited more than 20% after the incubation for 30 min at 90^0C although that of free one was hardly observed. Moreover, the half-life of BCP-adsorbed HEWL was 13 min at 90^0C while that of free one was 4 min.

Keywords: Adsorption, Biochar, Hen Egg White Lysozyme, Thermal Stabilization

1. Introduction

The application of biomass materials, which are carbon neutral, to energies and functional materials is crucial to resolve serious global warming problems since conventional industrial processes tend to produce greenhouse gases such as CO_2 (Straathof, 2014; IPCC, 2014; Olivier et al., 2017). However, forestry residues have scarcely been utilized in the industrial field although an enormous amount of forestry residues has been discharged in the world. 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.

Bioprocesses such as biotransformation, biosensor, biofuel cell, and so on have been widely studied as sustainable processes (Heinzle et al., 2006). Bioprocesses have generally utilized enzymes, which are biocatalysts that exhibit outstanding biological activity and specificity under mild conditions (Sheldon & Woodley, 2018; Buchholz et al., 2012; Leech et al., 2012; Silwana et al., 2014). Enzymes are generally stable in a cell. However, they are gradually denatured and inactivated under various physical and chemical stresses such as heat, organic solvents, and so on (Bailey & Ollas, 1986). Moreover, enzymes are expensive since they are obtained from living bodies by complicated separation processes. In order to enhance the stability of enzymes used in vitro and recycle enzymes, enzyme immobilization has been carried out mainly by attaching enzymes to solid carriers (Zdata et al., 2018).

In order to apply forestry residues to enzyme carriers, we have so far examined the catalysis of enzymes adsorbed on bamboo charcoal powder (BCP), which was prepared from forestry residues by pyrolysis. We have reported that the adsorption of enzymes on BCP markedly improve the catalytic activity of enzymes in organic solvents (Noritomi et al., 2017a; Noritomi et al., 2017b; Noritomi et al., 2018). In this study, we have examined the stability of enzymes adsorbed on BCP in an aqueous solution at high temperatures. As a model enzyme, hen egg white lysozyme (HEWL) has been employed, since it is well investigated regarding its structure, properties, functions, and applications such as an enzyme-sterilizing filter (Ahern & Klibanov, 1985; Jollès, 1996; Nohara et al., 1999; Tanaka et al., 2003).

2. Method

2.1 Materials

Lysozyme from hen egg white (EC 3.2.1.17, 46400 units/mg solid, MW=14300, pI 11) and *Micrococcus lysodeikticus* (ATCC No. 4698) were purchased from Sigma-Aldrich Co. (St. Louis, USA).

2.2 Preparation of Bamboo Charcoal Powder

To prepare bamboo charcoal as a biochar, under a nitrogen atmosphere, bamboo waste was dried at 180^0C for 2 h, was pyrolyzed at 450^0C for 2 h, was carbonized at 350^0C for 3 h, and then was cooled at 100^0C for 1 h by pyrolyzer (EE21 Pyrolyzer, EEN Co. Ltd., Japan) (Noritomi et al., 2017b). Bamboo charcoal powder (BCP) was obtained by grinding the resultant bamboo charcoal with a jet mill (100AS, Fuji Sangyo Co. Ltd., Japan).

2.3 Characterization of BCP

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

All samples were outgassed at 300^0C for 8 h prior to the nitrogen adsorption measurements. The specific surface area of BCP was obtained using a micropore system (BELSORP-mini II, BEL JAPAN, INC.), and the pore size distribution was calculated with the use of the micropore analysis method (MP method).

BCP was analyzed by solid-state DD/MAS ^{13}C-NMR spectroscopy (CMX300 Infinity, Chemagnetics, USA) operating at a ^{13}C resonance frequency of 75.188829MHz and magic angle spinning (MAS) with spin rate of 10.4kHz.

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 100μm.

The ζ potentials of BCP were measured by electrophoretic light scattering (ELS-Z2, OTSUKA Electronics Co. Ltd.).

2.4 Adsorption of HEWL onto BCP

As a typical procedure, 0.01M phosphate buffer solution of pH 7 containing 500μM hen egg white lysozyme (HEWL) and 3g/L BCP was incubated at 25^0C and 120rpm for 24hr. After adsorption, the mixture was filtrated with a membrane filter (pore size: 0.1μm, Millipore Co. Ltd., USA). The amount of HEWL adsorbed on BCP was calculated by subtracting the amount of HEWL included in the supernatant liquid after adsorption from the amount of HEWL in its aqueous solution before adsorption. The amount of HEWL was measured at 280nm by UV/vis spectrophotometer (UV-1800, Shimadzu Co. Ltd., Japan).

2.5 Heat Treatment of BCP-Adsorbed HEWL

A requisite amount of BCP-adsorbed HEWL was dispersed in 0.01 M phosphate buffer solution of pH 7.0, the mixture was incubated in a thermostated silicone oil bath at high temperatures for an adequate time, and then the mixture was cooled at 25^0C for 30min.

2.6 Measurement of Remaining Activity of HEWL

HEWL catalyzes the hydrolysis of the β-1,4 glycosidic linkage between the *N*-acetylmuramic acid and *N*-acetylglucosamine components of peptidoglycan in the cell membrane of bacteria. This causes the breakdown and removal of peptidoglycan from the bacterium which results in cell bursting or lysis in natural hypotonic solutions (Illanes, 1999; Noritomi et al., 2011). After the heat treatment, an aqueous solution of BCP-adsorbed HEWL was cooled in a thermostated water bath at 25°C for 30min. After 350μL of the cooled aqueous solution of BCP-adsorbed HEWL was added to 21mL of 0.01M phosphate buffer solution of pH 7 containing 200mg/L *Micrococcus lysodeikticus*, and then the mixture was incubated at 25°C with stirring, the absorbance of the mixuture was periodically measured at 450nm by UV/vis spectrophotometer (UV-1800, Shimadzu Co. Ltd.). Bacterial lysis obeys a first order reaction. The lysis rate constant (k) is calculated by

$$\ln (A^o_{450} / A_{450}) = kt \quad (1)$$

where t , A^o_{450}, and A_{450} are the reaction time, the absorbance of the substrate solution at 450nm at T=0, and the absorbance of the substrate solution at 450nm at T=t, respectively. The remaining activity (R. A.) is defined as

$$\text{R. A.} = 100 \times k / k_o \quad (2)$$

where k_o and k are the lysis rate constants at 25°C of BCP-adsorbed HEWL before and after heat treatment, respectively.

2.7 Measurements of ζ Potential and Fourier Transform Infrared (FTIR) Spectroscopy of HEWL

The ζ potentials of HEWL were measured by massively parallel-phase analysis light scattering (Möbiuζ, WYATT Technology Co. Ltd.)

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

3. Results and Discussion

3.1 Characterization of BCP and Adsorption of HEWL on BCP

In the present study, BCP was obtained by an environmentally benign process. Bamboo waste was pyrolyzed without burn at low temperatures, compared to the conventional charcoal production. Accordingly, carbon dioxide emission was reduced, the energy cost was low, and the atom economy of carbon was high.

Figure 1 shows the scanning electron micrograph of BCP. The surface state of BCP was almost smooth under the magnification measured in the present study. On the other hand, the specific surface area of BCP was 317m^2/g, which was similar to that of a conventional wood charcoal. The pore diameter peak of BCP was 0.8nm. This size is much smaller than that of HEWL since the size of HEWL is 4.5 x 3.0 x 3.0nm (Jollès, 1996).

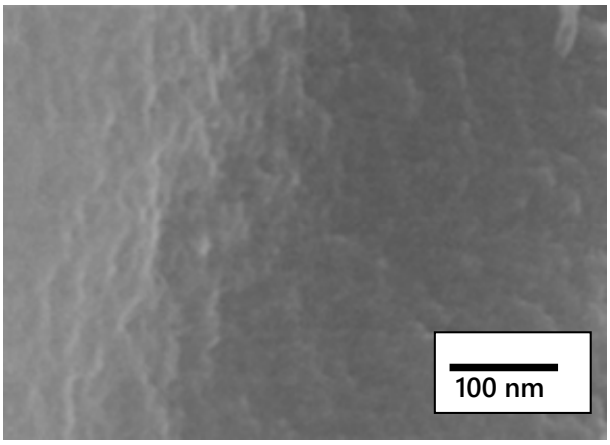

Figure 1. SEM image of bamboo charcoal powder (BCP)

As seen in Figure 2, carbonyl, carboxyl, and phenolic groups were detected by solid-state DD/MAS ^{13}C-NMR spectroscopy. Moreover, on the measurement of X-ray photoelectron spectroscopy (XPS) of BCP, C-O, O-C-O, C=O, COOH, C-N, and so on were detected. Functional groups containing oxygen atoms are formed by thermal decomposition of cellulose and hemicellulose at 500^{o}C, and functional groups decrease with increasing carbonization temperature (Asada et al., 2002; Nishimiya et al., 1998). Those functional groups are useful for adsorbing enzymes to solid carriers firmly (Zdata et al., 2018).

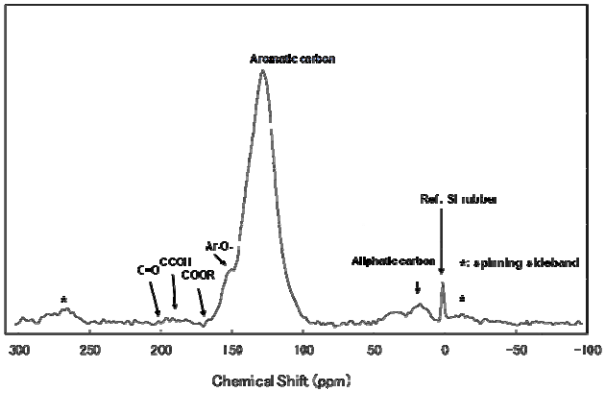

Figure 2. DD/MAS ^{13}C-NMR spectrum of bamboo charcoal powder (BCP)

HEWL was adsorbed on BCP at 9μmol/g. When it is assumed that HEWL molecules in the end-on orientation contact BCP with their major axis perpendicular to the surface of BCP or those in the side-on orientation contact BCP with their minor axis perpendicular to the surface of BCP, the coverage of end-on was 12% while that of

side-on was 19%. Consequently, it is suggested that HEWL is adsorbed at monolayer. Moreover, the ζ-potential of HEWL was 6.2mV whereas that of BCP was -53.5mV at pH 7, where the adsorption was carried out. These results indicate that the electrostatic interaction between the positively-charged HEWL and the negatively-charged surface of BCP mainly contributes to the adsorption of HEWL on BCP.

3.2 Conformation of BCP-Adsorbed HEWL

Three dementional structure of enzymes consists of secondary structures such as α-helix and β-sheet. In order to assess the structural change of HEWL by the adsorption of HEWL on BCP, we have measured the FTIR spectra of HEWL before and after the adsorption of HEWL on BCP.

Figure 3 shows the FTIR spectra of free and BCP-adsorbed HEWL. The spectral pattern of HEWL varied before and after the adsorption in the region of amide I (1700 – 1600cm^{-1}), which is due entirely to the C=O stretch vibrations of the peptide linkages (Surewicz & Mantsch, 1988). The band at ca. 1650cm^{-1} is assignable to α-helix while the band at ca. 1680cm^{-1} is assignable to intramolecular β-sheet. In Table 1, the ratio of the absorbance at 1681 cm^{-1} to the absorbance at 1647cm^{-1} of BCP-adsorbed HEWL was different from that of free one. This result indicates that the secondary structure of HEWL is altered to some extent by the adsorption of HEWL on BCP.

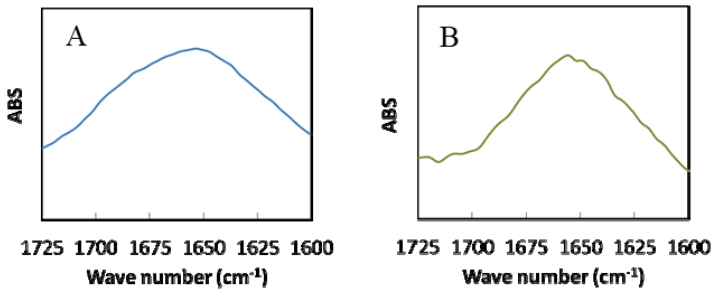

Figure 3. (A) FTIR spectrum of free HEWL. (B) FTIR spectrum of BCP-adsorbed HEWL

Table 1. Ratio of the absorbance at 1681cm^{-1} to the absorbance at 1647cm^{-1} (ABS_{1681}/ABS_{1647}) of free and biochar-adsorbed HEWL provided by the FTIR measurement

Samples	ABS_{1681}/ABS_{1647} (-)
Free HEWL	0.88
BCP-adsorbed HEWL	0.69

3.3 Influence of Incubation Temperature on Remaining Activity of BCP-Adsorbed HEWL

Modest heating causes proteins such as enzymes dissolved in an aqueous solution to be denatured and inactivated by the unfolding of proteins due to the disruption of weak interactions such as ionic bonds, hydrogen bonds, and hydrophobic interactions, which are prime determinants of protein tertiary structures (Klivanov, 1983; Creighton, 1989; Noritomi et al., 2011).

Figure 4 shows the relationship between the remaining activities of free and BCP-adsorbed HEWL and the incubation temperature after the incubation for 30 min. As seen in the figure, the dependence of the remaining activity on the incubation temperature exhibited the sigmoid curve. The remaining activity of free HEWL gradually dropped from 60ºC, and dramatically decreased from 70ºC while the remaining activity of BCP-adsorbed HEWL descended from 75ºC, and still exhibited 21% at 90ºC, which was about thirty times larger than that of free one. Accordingly, the thermal denaturation curve of BCP-adsorbed HEWL was shifted to high temperatures, compared to that of free one.

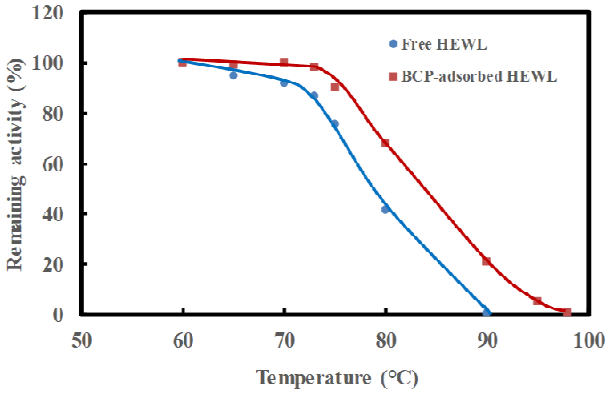

Figure 4. Thermal denaturation curves of free and BCP-adsorbed HEWL. The aqueous solution of free or BCP-adsorbed HEWL was incubated at a predetermined temperature for 30 min

3.4 Influence of Incubation Time on Remaining Activity of BCP-Adsorbed HEWL at High Temperatures

In general, the denaturation of enzymes progresses with the incubation time at high temperatures (Creighton, 1989). Figure 5 shows the time courses of remaining activities of free and BCP-adsorbed HEWL at 90°C. The remaining activities of free and BCP-adsorbed HEWL decreased with increasing the incubation time. Free HEWL solution immediately became turbid due to the aggregation of denatured enzymes as soon as the incubation started. On the other hand, no aggregation of enzymes was observed during the incubation in the solution of BCP-adsorbed HEWL, indicating that enzymes are firmly adsorbed on BCP even at high temperatures. In the figure, the relationship between the remaining activities of free and BCP-adsorbed HEWL and the incubation time could be correlated by first-order kinetics. As shown in Table 2, the half-life of BCP-adsorbed HEWL was more than three times longer than that of free one. Accordingly, the result indicates that the heat stress tolerance of HEWL is markedly enhanced by the adsorption of HEWL on BCP.

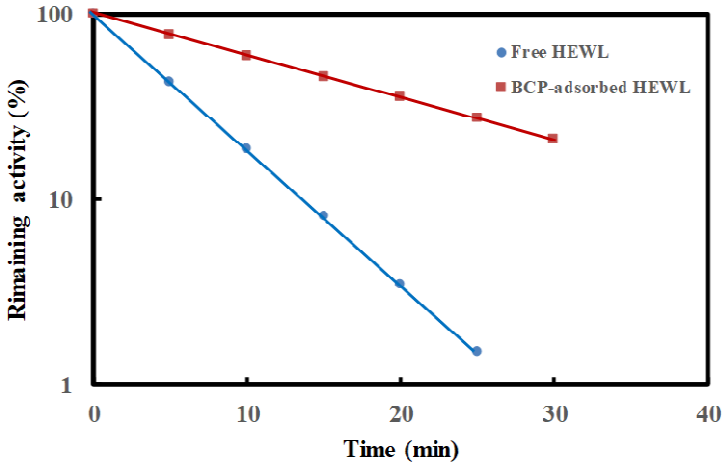

Figure 5. Time courses of remaining activities of free and BCP-adsorbed HEWL at 90^0C

Table 2. Rate constants and half-lives of denaturation of HEWL at 90^0C

Samples	Rate constant (min^{-1})	Half life (min)
Free HEWL	0.17	4
BCP-adsorbed HEWL	0.052	13

4. Conclusions

We have demonstrated that BCP remarkably improves the stability of HEWL at high temperatures as an enzyme carrier. HEWL was firmly adsorbed on BCP even at high temperatures. The conformation of HEWL changed to some extent by the adsorption of HEWL on BCP. The denaturation curve of BCP-adsorbed HEWL was shifted to high temperatures, compared to that of free one. The half-life of BCP-adsorbed HEWL was more than three times longer than that of free one at 90^{o}C. Accordingly, the results indicate that BCP is suitable as an enzyme carrior for the heat stress tolerance of enzymes.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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