International Journal of GEOMATE, July, 2016, Vol. 11, Issue 23, pp. 2123-2128
Geotec., Const. Mat. & Env., ISSN: 2186-2982(Print), 2186-2990(Online), Japan

EFFECT OF ADSORPTION CONDITION ON THERMAL STABILITY OF PROTEINS ADSORBED ONTO BIOMASS CHARCOAL POWDER

Hidetaka Noritomi[1], Shunichi Kurihara[1], Nobuyuki Endo[2], Satoru Kato[1] and Katsumi Uchiyama[1]

[1]Department of Applied Chemistry, Tokyo Metropolitan University, Japan; [2] EEN Co., Ltd., Japan

ABSTRACT: Authors have found out that bamboo charcoal powder (BCP), which is prepared from bamboo wastes by pyrolysis at low temperatures, is very useful as a carrier for the thermal stabilization of proteins. Hen egg white lysozyme (HEWL) and α-chymotrypsin (CT) were effectively adsorbed onto BCP. The thermal stability of BCP-adsorbed HEWL was strongly dependent upon the adsorption conditions such as solution pH, ionic strength, and temperature. Moreover, writers have revealed the heat-resistant mechanism on the basis of the structure of HEWL. Likewise, the thermal stability of BCP-adsorbed CT could be enhanced by selecting the adsorption condition.

Keywords: Biomass Charcoal Powder, Protein, Adsorption, Thermal Stability, Hen Egg White Lysozyme, α-Chymotrypsin

1. INTRODUCTION

The utilization of biomass to energies and functional materials is one of the most important challenges to establish a recycling society [1]-[4]. However, plant biomass wastes have not sufficiently been recycled yet, although a large number of plant biomass wastes have been discharged in the world. Moreover, the development in the high value-added function of plant biomass wastes has been desired.

On the other hand, proteins are biomolecules of great importance in the fields of biotechnology, fine chemistry, pharmacy, biosensor, and biofuel cell, since they exhibit their outstanding biological activities under mild conditions [5]-[7]. However, most of proteins are immediately denatured and inactivated by heat due to the disruption of weak interactions including ionic bonds, hydrogen bonds, and hydrophobic interactions, which are prime determinants of protein tertiary structures [8]. Adsorption of proteins onto various water-insoluble carriers has attracted continuous attention as the simplest and most economical method of stabilizing proteins [9], [10]. Moreover, the performances of adsorbed proteins such as activity, specificity, and stability are markedly dependent upon the physical and chemical surface properties of carriers. Accordingly, it is possible to derive the desired performances of proteins by selecting a suitable carrier. However, there have been few reports about the relation between the performances of adsorbed proteins and the adsorption conditions.

In order to investigate the valuable function of plant biomass wastes, authors have so far studied the interaction of proteins with biomass charcoal powders derived from plant biomass wastes by pyrolysis at low temperatures. Writers have found out that proteins are effectively adsorbed onto biomass charcoal powders, and adsorbed proteins exhibit the enhanced storage stability and the excellent thermal stability, compared to those of free proteins [11]-[15].

In the present work, researches have investigated the thermal stability and structure of proteins adsorbed onto bamboo charcoal powder (BCP) under various adsorption conditions such as ionic strength, solution pH, and temperature in order to address how the adsorption conditions affect the thermal stability of BCP-adsorbed proteins.

2. MATERIALS AND METHODS

2.1 Materials

Lysozyme from hen egg white (EC 3.2.1.17, 46400 units/mg solid, MW=14300, pI=11), α-chymotrypsin from bovine pancreas (EC 3.4.21.1, 52 units/mg solid, MW=25000), and *Micrococcus lysodeikticus* (ATCC No. 4698) were purchased from Sigma-Aldrich Co. (St. Louis, USA). *p*-Nitrophenyl acetate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Preparation of BCP

Under nitrogen atmosphere, bamboo wastes were dried at 180 ℃ for 2 hr, were pyrolyzed at 450 ℃ for 2 hr, were carbonized at 350 ℃ for 3 hr, and then were 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 Adsorption of Proteins onto BCP

As a typical procedure, 0.01 M phosphate buffer solution at pH 7 containing 500 μM hen egg white lysozyme (HEWL) and 3 g/L BCP was incubated at 25 ℃ and 120 rpm for 24 hr [11]. After adsorption, the mixture was filtrated with a membrane filter (pore size: 0.1 μm, 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 280 nm by UV/vis spectrophotometer (UV-1800, Shimadzu Co. Ltd., Japan).

Similarly, the adsorption of α-chymotrypsin (CT) onto BCP was carried out by using 300 μM CT instead of 500 μM HEWL [12].

2.4 Measurement of Activity of BCP-Adsorbed Proteins

The activity of HEWL was determined using *Micrococcus lysodeikticus* as a substrate [13]. Three hundred and fifty μL of 0.01 M phosphate buffer solution at pH 7.0 of BCP-adsorbed HEWL was added to 21 mL of 0.01 M phosphate buffer solution at pH 7.0 containing 200 mg/L *Micrococcus lysodeikticus*, and the mixture was incubated by stirring at 25 ℃. The absorbance of the mixture was periodically measured at 450 nm by UV/vis spectrophotometer (UV-1800, Shimadzu Co. Ltd.).

The activity of CT was determined using *p*-nitrophenyl acetate as a substrate [15]. Four mL of 0.01 M phosphate buffer solution at pH 7.5 of BCP-adsorbed CT was added to 16 mL of 0.01 M phosphate buffer solution at pH 7.5 containing 750 μM *p*-nitrophenyl acetate, and the mixture was incubated at 25 ℃ and 120 rpm. The absorbance of the mixture was periodically measured at 400 nm by UV/vis spectrophotometer.

2.5 Heat Treatment of BCP-Adsorbed Proteins

A requisite amount of BCP-adsorbed HEWL was dispersed in 0.01 M phosphate buffer solution at pH 7.0, the mixture was incubated in thermostated silicone oil bath at 90 ℃ for 30 min, and then was cooled at 25 ℃ for 30 min. On the other hand, a requisite amount of BCP-adsorbed CT was dispersed in 0.01 M phosphate buffer solution at pH 7.5, the mixture was incubated in thermostated water bath at 45 ℃ for 10 min, and then was cooled at 25 ℃ for 60 min.

In order to assess the thermal stability of BCP-adsorbed proteins, the activities of BCP-adsorbed proteins were measured before and after heat treatment. The remaining activity was obtained by Eq. (1).

$$\text{Remaining activity (\%)} = \frac{\text{Activity after heat treatment}}{\text{Activity before heat treatment}} \text{X } 100 \qquad (1)$$

2.6 Measurements of Circular Dichroism (CD), Fourier Transform Infrared (FTIR) Spectroscopies, and ζ -Potential

CD measurements of HEWL were carried out using a Jasco spectropolarimeter model J-820. The CD spectra were run on the HEWL solutions of 0.1 mg/mL in a quartz cell with 1.0 cm path length at an appropriate temperature.

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

The ζ potentials for HEWL were measured by massively parallel-phase analysis light scattering (Möbiu ζ , WYATT Technology Co. Ltd.), while those for BCP were measured by electrophoretic light scattering (ELS-Z2, OTSUKA Electronics Co. Ltd.).

3. RESULTS AND DISCUSSION

3.1 Effect of Ionic Strength of Adsorption Medium on Thermal Stability of BCP-Adsorbed HEWL

In order to investigate the relation between the thermal stability of BCP-adsorbed proteins and the adsorption condition, researches employed hen egg white lysozyme (HEWL) as a model protein, since it is well investigated regarding its structure, functions, and properties [16].

Researches have previously reported that the amount of proteins adsorbed onto BCP is strongly dependent upon the ionic strength of adsorption medium [11], [12]. So as to estimate the influence of ionic strength of adsorption medium on the thermal stability of BCP-adsorbed HEWL, authors have carried out the adsorption of HEWL onto BCP at pH 7 and different KCl concentrations. Figure 1 shows the relationship between the KCl concentration of adsorption medium and the remaining activity of BCP-adsorbed HEWL. The remaining activity of BCP-adsorbed HEWL decreased with an increase in KCl concentration. From the results of CP/MAS ^{13}C-NMR and X-ray photoelectron spectroscopy (XPS), acidic functional groups such as phenols, carbonyl groups, and carboxyl groups were detected in BCP [11]. On the other hand, Figure 2 shows the ζ

potentials of BCP and HEWL against the solution pH. As shown in Fig. 2, the ζ potential of BCP exhibited a negative value at pH 7, while the ζ potential of HEWL exhibited a positive value at pH 7. Accordingly, it is suggested that at higher ionic strength, the electrostatic attraction between HEWL and BCP decreases with a concomitant increase in the electrostatic screening effect when HEWL is adsorbed onto BCP.

Writers have measured the FTIR spectra of native and BCP-adsorbed HEWL to elucidate the influence of adsorption on the secondary structure of HEWL. Figure 3 shows the FTIR spectra of native HEWL and HEWL adsorbed onto BCP with or without KCl of adsorption medium. The most sensitive spectral region to protein secondary structural components is amide I (1700 – 1600 cm^{-1}), which is due almost entirely to the C=O stretch vibrations of the peptide linkages [17]. As seen in Fig. 3, the spectral pattern of BCP-adsorbed HEWL exhibited a specific shape of secondary structures,

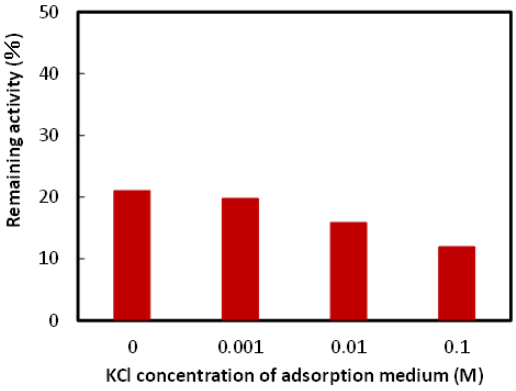

Fig. 1 Effect of KCl concentration of adsorption medium on the remaining activity of BCP-adsorbed HEWL after the heat treatment at 90 ℃ for 30 min.

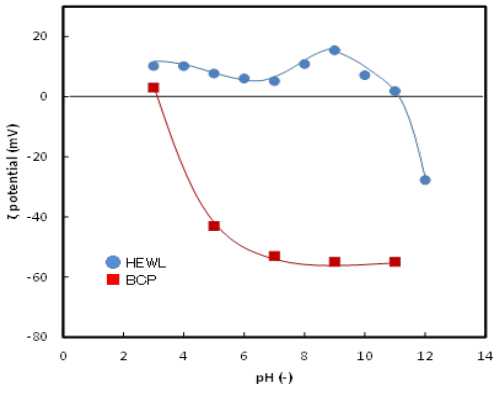

Fig. 2 Relationship of ζ-potentials of HEWL and BCP with solution pH.

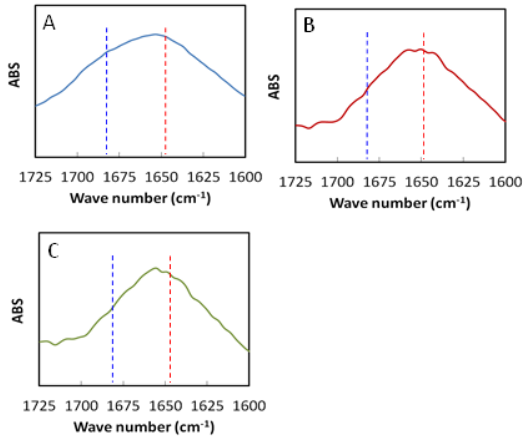

Fig. 3 (A) FTIR spectrum of free HEWL. (B) FTIR spectrum of HEWL adsorbed onto BCP without KCl. (C) FTIR spectrum of HEWL adsorbed onto BCP at 0.1 M KCl.

similar to native HEWL. Thus, the secondary structure of HEWL was kept to some extent after the adsorption with or without KBr. In order to evaluate the change in the secondary structure of BCP-adsorbed HEWL, authors have assessed the ratio of the absorbance at 1681 cm^{-1} to the absorbance at 1647 cm^{-1} (ABS_{1681}/ABS_{1647}), since the band located at ca. 1681cm^{-1} is assigned to intramolecular β-sheet, and the band located at ca. 1647 cm^{-1} is assigned to α-helix [17]. The ABS_{1681}/ABS_{1647} ratio at 0.1 M KCl (0.66) was similar to that without KCl (0.69) although the ABS_{1681}/ABS_{1647} ratio of BCP-adsorbed HEWL was different from that of native HEWL (0.88). These results indicate that the change in the remaining activity of BCP-adsorbed HEWL results not from the structural change of BCP-adsorbed HEWL but from the change in the electrostatic attraction between BCP and HEWL due to the electrostatic screening effect. In other words, when the structures of HEWL adsorbed onto BCP under different conditions are similar, the thermal stability of BCP-adsorbed HEWL is more efficiently enhanced by the stronger electrostatic attraction between HEWL and BCP.

3.2 Effect of pH of Adsorption Medium on Thermal Stability of BCP-adsorbed HEWL

In general, the activity and stability of proteins in an aqueous solution are influenced by the solution pH [5]. Figure 4 shows the relation between the solution pH of adsorption medium and the remaining activity of HEWL adsorbed onto BCP. The remaining activity of BCP-adsorbed HEWL was markedly dependent upon the pH of adsorption medium, and exhibited the maximum at pH 5. The electrostatic force between HEWL and BCP at pH 5 is smaller than that in the range from pH 7 to 9 due

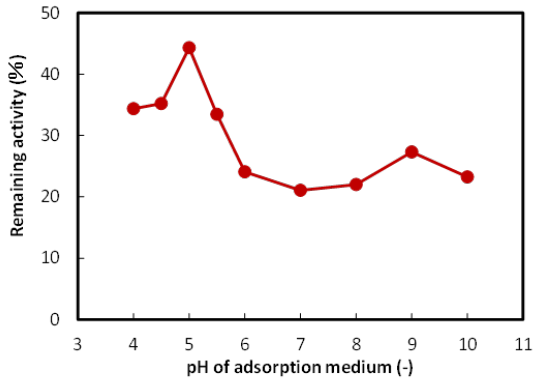

Fig. 4 Effect of pH of adsorption medium on the remaining activity of BCP-adsorbed HEWL after the heat treatment at 90 ℃ for 30 min.

to ζ potentials of BCP and HEWL, as shown in Fig. 2. In this case, the remaining activity of BCP-adsorbed HEWL was not correlated with the electrostatic force between HEWL and BCP.

Writers have measured the CD spectra of HEWL at different pH to investigate the influence of solution pH on the conformation of HEWL, since a protein is a polyelectrolyte. Figure 5 shows the CD spectra of free HEWL in the far- and near-UV regions dissolved in aqueous solutions at different pH. In the far-UV region, the CD spectrum of HEWL was independent upon the solution pH. The

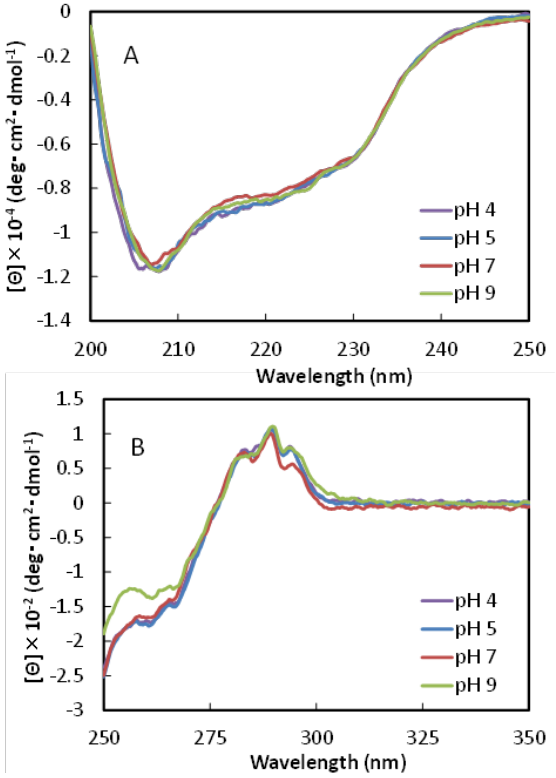

Fig. 5 (A) CD spectra of HEWL dissolved in buffer solutions at different pH in far-UV region. (B) CD spectra of HEWL dissolved in buffer solutions at different pH in near-UV region.

CD spectra in the far-UV (200-250 nm) correspond upon the secondary structure of proteins [18], [19]. Especially, the mean residue ellipticity at 215 nm is assigned to β-sheet, and the mean residue ellipticity at 222 nm is assigned to α-helix. The constancy in the mean residue ellipticities at 215 and 222 nm at different pH indicates that the β-sheet and α-helix contents of HEWL are kept constant at different pH. Likewise, the CD spectrum of HEWL in the near-UV region did not almost change with the solution pH. The CD spectra in the near-UV (250-350 nm) correspond upon the local asymmetric environment of aromatic amino acid residues [20], [21]. The mean residue ellipticities at 283 and 289 nm are assigned to tryptophan and tyrosine residues, and the mean residue ellipticity at 294 nm is assigned to tryptophan. Accordingly, the result of CD spectra in the near-UV suggests that the tertiary structure of HEWL is not influenced by the solution pH in the present work.

Figure 6 shows the FTIR spectra of native HEWL and HEWL adsorbed onto BCP at different pH. The spectral pattern of BCP-adsorbed HEWL was influenced by the pH of adsorption medium. The ABS_{1681}/ABS_{1647} ratio at pH 5 (0.86), where the remaining activity showed the maximum value, was similar to that of native HEWL (0.88). Likewise, the

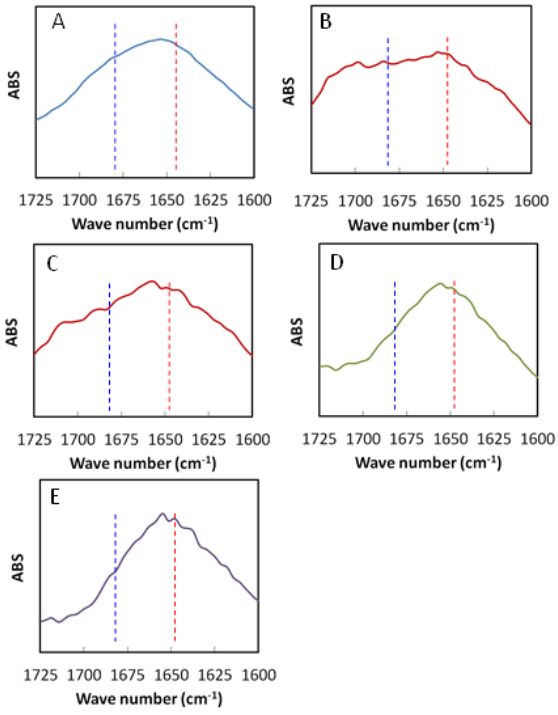

Fig. 6 (A) FTIR spectrum of free HEWL. (B) FTIR spectrum of HEWL adsorbed onto BCP at pH 4. (C) FTIR spectrum of HEWL adsorbed onto BCP at pH 5. (D) FTIR spectrum of HEWL adsorbed onto BCP at pH 7. (E) FTIR spectrum of HEWL adsorbed onto BCP at pH 9.

ABS_{1681}/ABS_{1647} ratio at pH 4 (0.92) was near that of native HEWL. On the other hand, the ABS_{1681}/ABS_{1647} ratios at pH 7 (0.69) and 9 (0.61), where the electrostatic force between HEWL and BCP was high due to ζ potentials of BCP and HEWL, were different from that of native HEWL. From these results, authors have summarized about the effect of solution pH of adsorption medium on the thermal stability of BCP-adsorbed HEWL as follows. When HEWL was adsorbed onto BCP at pH 4, the structure of BCP-adsorbed HEWL was nearly the native structure of HEWL, but the electrostatic force between HEWL and BCP was not sufficient for the thermal stability. When HEWL was adsorbed onto BCP at pH 5, the native structure of HEWL was kept, and the electrostatic force between HEWL and BCP was strong enough to help retain the structure of HEWL at high temperatures. When HEWL was adsorbed onto BCP at pH 7 and 9, the structure of HEWL was partially destroyed since the electrostatic force was too strong to keep the native structure of HEWL, and the thermal stability of BCP-adsorbed HEWL dropped.

3.3 Effect of Temperature of Adsorption Medium on Thermal Stability of BCP-Adsorbed HEWL

The activity and stability of proteins tend to depend upon the temperature [5]. Furthermore, the writers have found out that the temperature markedly affects the amount of proteins adsorbed onto BCP [11], [12].

In order to estimate the dependence of the thermal stability of BCP-adsorbed HEWL on the temperature of adsorption medium, the researches have examined the adsorption of HEWL onto BCP at pH 7 and different temperatures. Figure 7 shows the relationship between the temperature of adsorption medium and the remaining activity of BCP-adsorbed HEWL. The remaining activity of BCP-adsorbed HEWL decreased with increasing the temperature of adsorption medium. On the other hand, the CD spectrum of HEWL remained almost unchanged with the temperature both in the far- and near-UV regions, indicating that the conformation of HEWL is not influenced by heat in the present temperature range. Moreover, the FTIR spectral patterns and the ABS_{1681}/ABS_{1647} ratios of BCP-adsorbed HEWL at 5, 25, and 50 ℃ were similar, indicating that the secondary structure of BCP-adsorbed HEWL is not dependent upon the temperature of adsorption medium. It has been reported that the orientation of protein molecules onto the solid surface is influenced by the temperature [22]. It is suggested that the orientation of HEWL adsorbed onto BCP at lower temperature is more beneficial to the thermal stability of BCP-adsorbed HEWL.

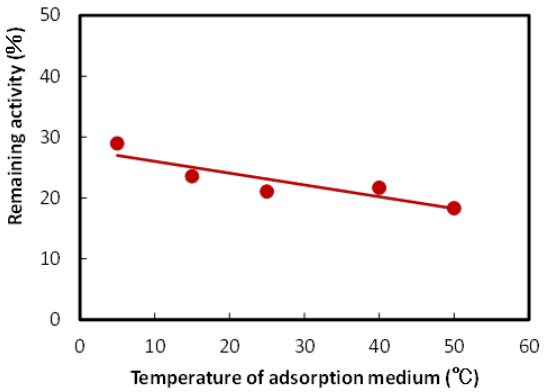

Fig. 7 Effect of temperature of adsorption medium on the remaining activity of BCP-adsorbed HEWL after the heat treatment at 90 ℃ for 30 min.

3.4 Effect of Adsorption Condition on Thermal Stability of BCP-Adsorbed CT

In order to assess the generality on the effect of adsorption condition on the thermal stability of BCP-adsorbed proteins, the researches employed bovine pancreas α-chymotrypsin (CT) as another model protein, since it is well investigated regarding its structure, functions, and properties [23].

The remaining activity of CT adsorbed onto BCP at pH 5 and 15 ℃ (65%) was 1.2 times greater than that at pH 7 and 25 ℃ (55%). The result indicates that the selection of adsorption condition is remarkably effective in enhancing the thermal stability of BCP-adsorbed CT, similar to the case of BCP-adsorbed HEWL.

4. CONCLUSION

The authors have demonstrated that the thermal stability of BCP-adsorbed proteins varies accordingly to the history of adsorption. The thermal stability of BCP-adsorbed HEWL increased with a decrease in the ionic strength of adsorption medium. The thermal stability and structure of BCP-adsorbed HEWL were strongly dependent upon the pH of adsorption medium, and the maximum remaining activity was obtained at pH 5, where the structure of BCP-adsorbed HEWL was kept native. The thermal stability of BCP-adsorbed HEWL increased with decreasing the temperature of adsorption medium. Likewise, the thermal stability of BCP-adsorbed CT was enhanced by selecting the adsorption condition. These results indicate that BCP-adsorbed proteins exhibit the excellent thermal stability when the native structure of proteins is kept after the adsorption, and the adsorption force is strong enough to help retain its structure against the heat stress. Accordingly, the enhancement in the thermal stabilization of proteins by selecting the adsorption condition would be encouraging for the preparation

of immobilized enzyme, biosensor, and biofuel cell.

5. ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (C) (No. 24561013).

6. REFERENCES

[1] Ho YC, Show KY, "A perspective in renewable energy production from biomass pyrolysis-challenges and prospects", Current Organic Chem., Vol. 19, 2015, pp. 423-436.

[2] Straathof AJ, "Transformation of biomass into commodity chemicals using enzymes or cells", Chem. Rev., Vol. 114, 2014, pp. 1871-1908.

[3] Peterson SC, Jackson MA, Appell M, "Biochar: Sustainable and versatile", ACS Symp. Series, Vol. 1143, 2013, pp. 193-205.

[4] Manya JJ, "Pyrolysis for biochar purposes: A review to establish current knowledge gaps and research needs", Environmental Sci. Technol., Vol. 46, 2012, pp. 7939-7954.

[5] Buchholz K, Kasche V, Bornscheuer UT, Biocatalyst and Enzyme Technology 2nd ed. Wiley-Blackwell, 2012.

[6] Silwana B, Horst CVD, Iwuoha E, "Amperometric determination of cadmium, lead, and mercury metal ions using a novel polymer immobilized horseradish peroxidase biosensor system", J. Environmental Sci. Health Part A, Vol. 49, 2014, pp.1501-1511.

[7] Leech D, Kavanagh P, Schuhmann W, "Enzymatic fuel cells: Recent progress", Electrochimica Acta, 84, 2012, pp. 223-234.

[8] Volkin DB, Klibanov AM, "Minimizing protein inactivation", Protein Function: Practical Approach, Creighton Ed. IRL Press, 1989, pp. 1-24.

[9] Elnashar MMM, "Review article: Immobilized molecules using biomaterials and nanobiotechnology", J. Biomaterials Nanobiotechnol., Vol. 1, 2010, pp. 61-77.

[10] Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lorente R, "Improvement of enzyme activity, stability and selectivity via immobilization techniques", Enzyme Microbial Technol., Vol. 40, 2007, pp. 1451-1463.

[11] Noritomi H, Iwai D, Kai R, Tanaka M, Kato S, "Adsorption of lysozyme on biomass charcoal powder prepared from plant biomass wastes", J. Chem. Eng. Jpn., Vol.v46, 2013, pp. 196-200.

[12] Noritomi H, Hishinuma K, Kurihara S, Nishigami J, Takemoto T, Endo N, Kato S, "Adsorption of α-chymotrypsin on plant biomass charcoal", J. Surface Eng. Materials Adv. Technol., Vol. 3, 2013, pp. 269-274.

[13] Noritomi H, Kai R, Iwai D, Tanaka H, Kamiya R, Tanaka M, Muneki K, Kato S, "Increase in thermal stability of proteins adsorbed on biomass charcoal powder prepared from plant biomass wastes", J. Biomedical Sci. Eng., Vol. 4, 2011, pp. 692-698.

[14] Noritomi H, Ishiyama R, Kai R, Iwai D, Tanaka M, Kato S, "Immobilization of lysozyme on biomass charcoal powder derived from plant biomass wastes", J. Biomaterials Nanobiotechnol., Vol 3, 2012, pp. 446-451.

[15] Noritomi H, Kurihara S, Endo N, Kato S, "Heat-resistant properties of α-chymotrypsin adsorbed onto biomass charcoal powder", J. Biomaterials Nanobiotechnol., Vol. 5, 2014, pp. 179-185.

[16] Jollès P, Lysozymes: Model Enzymes in Biochemistry and Biology 1st ed. Birkhäuser Verlag, 1996.

[17] Surewicz WK, Mantsch HH, "New insight into protein secondary structure from resolution-enhanced infrared spectra", Biochim. Biophys. Acta, Vol. 952, 1988, pp. 115-130.

[18] Chen YH, Yang JT, Martinez HM, "Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion", Biochemistry, Vol. 11, 1972, pp. 4120-4131.

[19] Venyaminov SY, Yang JT, "Determination of protein secondary structure", Circular Dichroism and the Conformational Analysis of Biomolecules, Fasman Ed. Plenum Press, 1996, pp. 69-107.

[20] Ikeda K, Hamaguchi K, "The binding of *N*-acetylglucosamine to lysozyme: Studies on circular dichroism", J. Biochem., Vol. 66, 1969, pp. 513-520.

[21] Woody RW, Dunker AK, "Aromatic and cysteine side-chain circular dichroism in proteins", Circular Dichroism and the Conformational Analysis of Biomolecules, Fasman Ed. Plenum Press, 1996, pp. 109-157.

[22] Cheng X, Canavan HE, Graham DJ, Castner DG, Ratner BD, "Temperature dependent activity and structure of adsorbed proteins on plasma polymerized *N*-isopropyl acrylamide", Biointerphases, Vol. 1, 2006, pp.61-72.

[23] Kumar A, Venkatesu P, "Overview of the stability of α-chymotrypsin in different solvent media, Chem. Rev., 112, 2012, pp.4283-4307.

International Journal of GEOMATE, July, 2016, Vol. 11, Issue 23, pp. 2123-2128.
MS No. 1128 received on July 28, 2015 and reviewed under GEOMATE publication policies.

Corresponding Author: Hidetaka Noritomi