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Aggregation Structure and Functional Properties of (Inorganic Nanofiber/Pepsin) Hybrid Hydrogel

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Imogolite is a natural tubular aluminosilicate with an outer diameter of 2.5 nm and length of several micrometers. In this study, the gelation of imogolite aqueous solution by immobilization of pepsin was investigated. Hydrogel could be formed due to the electrostatic interaction between imogolite and pepsin as well as interaction between Al-OH groups of imogolite surface and the phosphoryl group of pepsin. The immobilization of pepsin onto imogolite was confirmed by infrared spectroscopy and thermogravimetry. It was revealed that the pepsin was successfully immobilized without denaturation. The dispersion state of pepsin in the nanofiber hydrogel and the 3-dimensional nanofiber network structure of imogolite hydrogel were observed by confocal scanning laser microscopy and field-emission scanning electron microscopy. The immobilized pepsin in the hybrid hydrogel retained 26 % of enzyme activity compared to free pepsin because of the inhibition of substrate diffusion into the 3D-network structure of the hydrogel. On the other hand, it was possible to react repeatedly with substrate and the thermal stability of the immobilized pepsin was improved compared with that of free pepsin.

Key words: Inorganic nanofiber, Pepsin, Hybrid hydrogel, Immobilized enzyme, Enzyme

1. INTRODUCTION

Imogolite, a hydrous aluminosilicate was discovered in the clay fraction of a glassy volcanic ash soil ("Imogolayer") of Kyushu, Japan in 1962 [1]. Fig. 1 shows the schematic representation of the structure of imogolite. Imogolite forms hollow nanotube with external diameter of ca. 2.5 nm and an internal diameter of 1 nm and length of several hundred nanometers to several micrometers, which has the general formula of $Al_2O_3 \cdot SiO_2 \cdot 2H_2O$ [2], and imogolite has high specific surface area. The outer surface of imogolite is composed of Al-OH groups and it can be therefore charged depending on the pH of the solution. Due to the electrostatic repulsion of outer surface, isolated units can form nanofibers in acidic solution (pH below 5). The Al-OH groups of the outer surface of imogolite interact specifically with phosphate ion or phosphoryl group [3, 4]. By utilizing the immobilization of the enzyme which has a phosphoryl group onto the nanofiber, it is possible to achieve the large amount of enzyme immobilization [5]. Furthermore, the enzyme activity can be expected to be maintained by forming the hybrid hydrogel immobilized with pepsin. The hybrid hydrogel also might form the structure which is dynamically and thermally more stable compared to conventional organic gel since imogolite which composes the hybrid hydrogel is an inorganic substance. In this paper, the authors report the preparation of the hybrid hydrogel by the immobilization of pepsin as a model enzyme with phosphoryl group and evaluated its aggregation structure and enzyme activity.

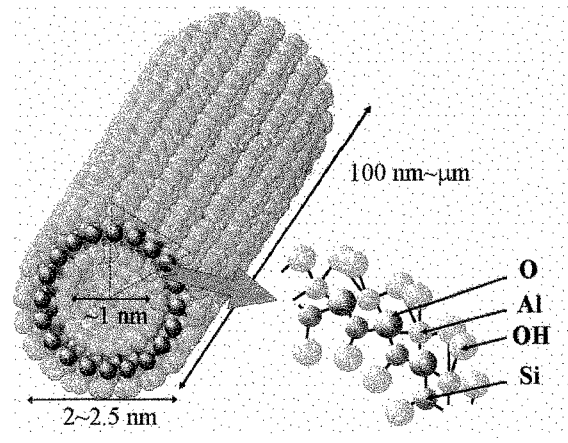

Fig.1 Schematic representation of the structure of imogolite.

Pepsin is an enzyme essential to the digestion process in animals. The optimal pH for its activity is 2.0. The enzyme is composed of 326 amino acid residues. One phosphoryl group is attached to the serine of the 68th residue in the native porcine pepsin [6]. The immobilization of pepsin would be possible since the crystal structure of pepsin revealed the presence of the phosphoryl group of serine-68 is at the surface of pepsin.

Moreover, the position of serine-68 is away from catalytic active site composed of asparaginic acid units of the 32nd and 215th residues [7]. The position of the phosphoryl group may not therefore affect with enzyme activity after immobilization.

The purpose of this study is preparation and evaluation of aggregation structure and enzyme activity of (imogolite / pepsin) hybrid hydrogel.

2. EXPERIMENTAL

2.1 Materials

Raw material of imogolite was collected from the weathered pumice bed in Kitakami area, Iwate, Japan. Imogolite purified from contamination in the raw materials was dispersed in acidic solution (pH = 3.1) by sonication. Porcine pepsin (2970 unit/mg) was obtained from Worthington Biochemical Co. and bovine serum hemoglobin and trichloroacetic acid were purchased from Wako Pure Chemical Industries, Co. Ltd. Fluorescein-5-isothiocyanate (FITC) and adenosine 5'-triphosphate, Alexa Fluor® 647 2'-(or-3')-O-(N-(2-aminoethyl)urethane), hexa(triethylammonium) salt (Alexa Fluor® 647 ATP) were purchased from Molecular Probes.

2.2 Preparation of hybrid hydrogel immobilized with pepsin

Pepsin was dissolved in 5 mL of pH = 3.1 acetic acid solution (1 mg/mL). Then the solution was added to 5 mL of pH = 3.1 imogolite solution (0.5 mg/mL) and incubated at 37 °C by shaking 120 rpm for 4 h. The mixture was centrifuged at 3000 rpm for 15 min to separate the hybrid hydrogel from the excess water and rinsed with pH = 3.1 acetic acid solution.

2.3 Confirmation of immobilization of pepsin in hybrid hydrogel

Infrared (IR) spectroscopy was carried out with Spectrum One (Perkin Elmer Japan Co., Ltd.) with a resolution of 0.5 cm^{-1} at room temperature. IR data were collected by averaging 64 scans between 2000 and 450 cm^{-1}. A specimen for IR measurement was prepared with the freeze-dried hybrid hydrogel and prepared as a tablet under pressure with KBr powder.

2.4 Estimation of the amount of immobilized pepsin in hybrid hydrogel

Thermogravimetric analysis (TGA) was carried out with TG8120 (Rigaku Co., Ltd.) for the estimation of the amount of immobilized pepsin. The sample for TGA was prepared by heating the freeze-dried hybrid hydrogel in the toluene at 84 °C, which is azeotropic temperature between toluene and H_2O, and removing residual toluene in vacuo.

2.5 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) observation of hybrid hydrogel was performed with LSM 510 META (Carl Zeiss Co., Ltd.). The sample for CLSM observation was prepared with the hybrid hydrogel composed of pre-labelled imogolite and pepsin, where imogolite and pepsin were labeled with Alexa Fluor® 647 ATP and FITC, respectively. The labeled hybrid hydrogel was visualized by excitating FITC and Alexa Fluor® 647 ATP with the 488-nm spectral band of an argon laser and 633-nm spectral band of a helium/neon laser. These two kinds of fluorescence were detected with a long pass filter for the range of less than 505 nm and band pass filter for the range 650-710 nm, respectively.

2.6 Scanning electron microscopy

Field-emission scanning electron microscopy (FE-SEM) observation of hybrid hydrogel was carried out with S-4300SE (Hitachi Co., Ltd). The specimen for FE-SEM observation was prepared by pre-coating the freeze-dried hybrid hydrogel with OsO_4 on the silicon wafer.

2.7 Evaluation of enzyme activity

Each sample of hybrid hydrogel and free pepsin was mixed with 2.5 mL of 2.5 wt% hemoglobin solution (pH = 3.1) and incubated at 37 °C by shaking at 120 rpm for 10 min. Then, 5 mL of 5 wt% trichloroacetic acid solution was added to the mixture and incubated at 37 °C for 1 h. The solution was centrifuged at 3000 rpm for 5 min. The supernatant was filtered with syringe filter (0.5 μm). The filtrate was transferred to a quartz cuvette and the absorbance at 280 nm was measured by UV-vis spectroscopy with Lambda35 (Perkin Elmer Japan Co., Ltd.) to estimate the reaction rate, ΔA_{280}/min [8]. In order to investigate the change of enzyme activity in the repeated reaction, the following experiment was carried out. After ΔA_{280}/min of free and immobilized pepsin was compared, the hybrid hydrogel was separated from hemoglobin solution by decantation. Trichloroacetic acid was added to the hemoglobin solution separated from hybrid hydrogel and enzyme activity was evaluated as described above. The hybrid hydrogel was rinsed with pH = 3.1 acetic acid solution and mixed with fresh hemoglobin solution. The change of enzyme activity was evaluated by repetition of this process 4 times.

2.8 Thermal stability evaluation of immobilized pepsin

Hybrid hydrogel and free pepsin solution were incubated in a water bath for 30 min at set temperatures range from 50 to 80 °C. Then, enzyme solutions were transferred to an ice bath to stop the denaturation of the enzyme, and the residual enzyme activity in each solution was evaluated by UV-vis spectroscopy as described in 2.7.

3. RESULTS AND DISCUSSION

3.1 Preparation of hybrid hydrogel

The hybrid hydrogel which was immobilized with pepsin onto imogolite was obtained by centrifugation of the mixture of imogolite and pepsin solution. Hydrogel could be formed due to the electrostatic interaction between imogolite and pepsin as well as interaction between Al-OH groups of imogolite surface and the phosphoryl group of pepsin. The water content of hybrid gel was 99.7 %.

3.2 Characterization of immobilized pepsin in the hybrid hydrogel

Fig. 2 shows the IR spectra of imogolite, pepsin, and hybrid hydrogel. The two sharp absorptions at 995 and 940 cm^{-1} were assigned to the stretching vibration of

Si-O-Al in imogolite [9]. The two sharp absorptions at 1647 and 1537 cm^{-1}, corresponding to the amide I and II bands, respectively, are characteristic bands of pepsin [10]. The occurrence of immobilization of pepsin onto imogolite via interaction between imogolite and pepsin was confirmed since the absorptions from imogolite and pepsin were observed simultaneously. Furthermore, it was suggested that pepsin was not denatured because the position of absorption peaks from pepsin was not changed before and after immobilization. The amount of immobilized pepsin onto imogolite was estimated by thermogravimetric analysis. The maximum value was approximately 1.8 mg of pepsin per 1 mg of imogolite.

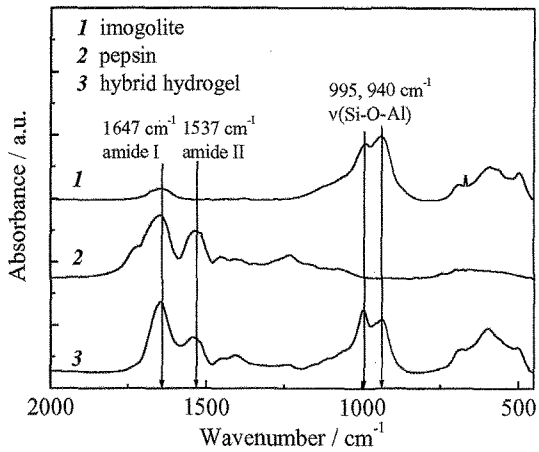

Fig. 2 IR spectra of imogolite, pepsin, and hybrid hydrogel immobilized with pepsin.

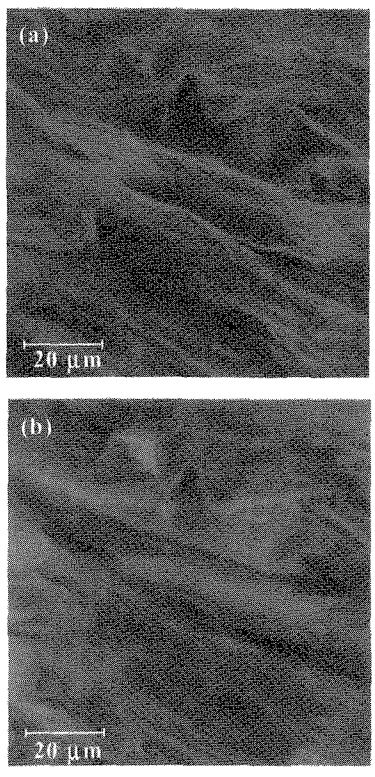

Fig. 3 CLSM image of (a) pepsin immobilized in the hybrid hydrogel and (b) imogolite. Pepsin and imogolite were labeled with FITC and Alexa Fluor® 647 ATP.

3.3 The dispersion state of pepsin in the hybrid hydrogel

The dispersion state of pepsin in the hybrid hydrogel was evaluated from CLSM image of pepsin in the hybrid hydrogel. Fig. 3 shows the CLSM image of pepsin immobilized in the hybrid hydrogel. Pepsin was finely dispersed in the hybrid hydrogel, and the fluorescent images of pepsin and imogolite showed the similar morphology. It was therefore speculated that pepsin in the hybrid hydrogel could be immobilized onto the imogolite.

3.4 Aggregation structure of hybrid hydrogel

For the evaluation of aggregated structure of hybrid hydrogel, FE-SEM observation was carried out. Fig. 4 shows the FE-SEM image of the hybrid hydrogel. Hybrid hydrogel was dehydrated by freeze-drying. The 3-dimentional network structure composed of imogolite was directly observed. The average pore size of the gel in this image was 108 nm.

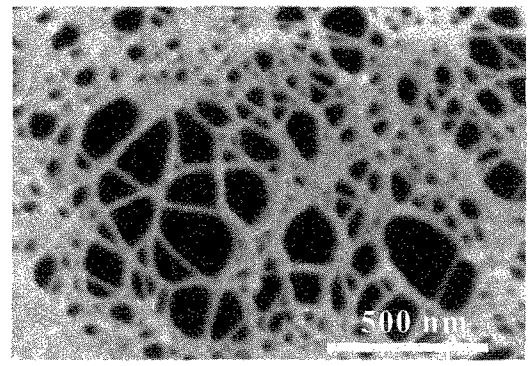

Fig. 4 FE-SEM image of hybrid hydrogel immobilized with pepsin onto imogolite.

3.5 Enzyme activity of immobilized pepsin

Table I summarized the enzyme activity for free and immobilized pepsin at 37 °C. ΔA_{280}/min stands for the reaction rate, i.e. enzyme activity. The enzyme activity

Table I ΔA_{280}/min of free and immobilized pepsin in the hybrid hydrogel

State of pepsin	ΔA_{280}/min
free	0.183
immobilized	0.048

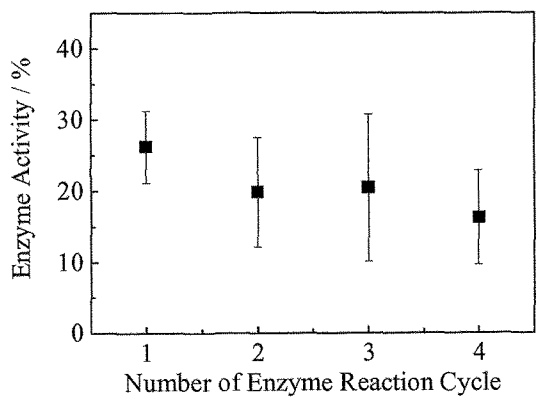

Fig. 5 Change of enzyme activity in the repeated reaction at 37 °C.

of the pepsin in the hybrid hydrogel retained ca. 26 % of enzyme activity compared to free pepsin. This decrease of enzyme activity was probably due to the inhibition of substrate diffusion into the 3-dimensional network structure of the hydrogel shown in Fig. 4 as well as inhibition of approach of substrate to the active site by the steric hindrance of imogolite. On the other hand, the immobilized pepsin can be expected to be easily recovered from the reaction system and to react with substrate repeatedly. We investigated the change of enzyme activity in the repeated reaction. Fig. 5 shows the change of enzyme activity in the repeated reaction. The enzyme activity of immobilized pepsin had a tendency to decrease as the number of reaction increased because the enzyme reaction progressed intermittently and small amount of pepsin might be detached from imogolite surface during enzyme reaction.

3.6 Thermal stability of immobilized pepsin in the hybrid hydrogel

Protein unfolding causes denaturation at higher temperature [11]. We investigated how the immobilization onto imogolite improves the thermal stability of enzyme activity, because immobilization may stabilize the protein structure at high temperature. Pepsin molecules in the hybrid hydrogel were found to have slightly higher thermal stability than free pepsin in aqueous solution. Indeed, the pepsin immobilized onto imogolite retained about 70 % of its activity after a 30-min incubation at 70 °C compared to 50 °C whereas free pepsin retained about 55 % under the same condition. The conformation and enzyme activity of an enzyme changes depending on the reaction temperature [12]. The improved thermal stability of enzyme activity for the immobilized pepsin is due to the increased conformational stability on immobilization.

4. CONCLUSION

The hybrid hydrogel immobilized with pepsin onto imogolite was prepared by a simple method with the interaction between pepsin and imogolite. The network structure of hybrid hydrogel was successfully observed. Although the enzyme activity of immobilized pepsin in the hybrid hydrogel was decreased, the immobilized pepsin could be reacted repeatedly after enzyme reaction. This method of immobilizing enzyme can be applicable to the preparation of hybrid hydrogel with other biomolecules which has or which was introduced phosphoryl groups. This (imogolite/pepsin) hybrid hydrogel is expected to apply to enzyme reaction with continuous flow method of substrate solution.

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