絹フィブロインによるグルコースオキシターゼの固定化機構

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Mechanism of glucose oxidase immobilization with silk fibroin

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Magnetic resonance spectroscopy, NMR and ESR, was applied for the examination of the interaction between glucose oxidase (GOD) and *Bombyx mori* silk fibroin, in order to analyze the superior attributes of silk fibroin for the immobilization of GOD. The ^{13}C NMR spectra and spin-lattice relaxation times, T_1, of *B. mori* silk fibroin in aqueous solution did not change in the presence of GOD. The spin-labeled silk fibroin membrane immobilizing GOD gave also the same ESR spectrum as that of the GOD-free membrane. In the presence of silk fibroin, the rate of the GOD reaction in aqueous solution monitored by ^{13}C NMR slightly decreased, although no peak broadening occurred, indicating a slight decrease in the diffusion of glucose and, as a result, a slight decrease in the contact between glucose and GOD rather than silk fibroin inhibition. In addition, no appreciable interaction was observed between the silk fibroin and 14 kinds of amino acids in the ^{13}C NMR spectra.

Recently, it has been demonstrated that silk fibroin is one of the superior biomaterials for glucose oxidase (GOD) immobilization, as indicated by the following experimental results (Kuzuhara *et al.*, 1987). 1) The high activity of the enzyme was maintained after enzyme-immobilization. 2) The enzyme leakage from the GOD-immobilized silk fibroin membrane was very small; only 0.05% after 36 days. 3) The stability of the enzyme against heat or pH changes was much improved. Such a superior enzyme-immobilizing ability of the silk fibroin implies that there is scarcely any appreciable interaction between the silk fibroin and the enzyme, i.e., the absence of silk fibroin inhibition. In order to verify this assumption and to use silk fibroin more widely as an enzyme-immobilization material, it was seemed necessary to examine the interaction between the enzyme and silk fibroin in detail.

This paper describes a magnetic resonance approach, NMR and ESR, to the study of the interaction between GOD and silk fibroin which was found to be negligible. In addition, the GOD reaction with glucose used as a substrate hardly changed in the presence of silk fibroin although the contact between glucose and GOD seemed to decrease slightly by the immobilization. There was no appreciable interaction between 14 kinds of amino acids and silk fibroin. These data indicate that silk fibroin is essentially a superior biomaterial for enzyme-immobilization.

Materials and Methods

Materials: The aqueous solution of *Bombyx*

mori silk fibroin was prepared according to the following procedures (Asakura *et al.* 1984). The cocoon from *B. mori* was degummed twice with a 0.5% Marseilles soap solution at 100°C for 0.5 hr and then washed with distilled water. After this degumming step, sericine, another silk protein, was removed from the cocoon and the silk fibroin protein was obtained. Then silk fibroin was dissolved in 9 M LiBr at 40°C. After dialysis against distilled water for 4 days, the solution was clarified by centrifugation at 10,000 rpm for 20～30 min. The supernatant was collected and brought gently up to the desired concentration with an electric fan.

The spin-labeled silk fibroin membranes containing GOD were obtained by casting the mixture of spin-labeled silk fibroin and GOD in aqueous solution. The spin-labeling method with a nitroxide radical for the tyrosine side chain of silk fibroin has been described elsewhere (Asakura *et al.* 1987).

Twenty kinds of amino acids and N-methylacetamide, NMA, were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. N-acetyl-L-alanine methylamide was synthesized in our laboratory (Asakura *et al.*, 1984). GOD (130 units/mg) and glucose were purchased from Wako Pure Chemical Industries, Ltd., Tokyo.

Measurements: ^{13}C NMR spectra of *B. mori* silk fibroin and the silk fibroin-GOD mixture in aqueous solution were measured at 25°C with a JEOL FX-90Q NMR spectrometer operating at 22.5 MHz equipped with a temperature controlle (Asakura, 1987). The concentrations of *B. mori* silk fibroin and GOD in the aqueous solution were 6.9 and 1.35% (w/v), respectively, and thus the GOD/silk fibroin ratio was 0.2, which was much higher than the usual ratio in the GOD-immobilized silk fibroin membrane (Kuzuhara *et al.*, 1987). The spin-lattice relaxation times, T_1, for the protonated carbons were measured by the inversion-recovery method, i.e., $180°\sim\tau\sim90°$ pulses, where τ is the delay time. The GOD reaction was observed in an aqueous solution with glucose as a substrate by ^{13}C NMR spectroscopy in the presence or absence of silk fibroin (Feather, 1970). In these experiments, the concentrations of GOD, glucose and *B. mori* silk fibroin were 6.8, 6.8 and 2.5% (w/v), respectively. In the ^1H and ^{13}C NMR observations of *B. mori* silk fibroin and several kinds of amino acid mixtures, the concentrations of silk fibroin and each amino acid were 4 and 1.7% (w/v), respectively. The NMR chemical shifts were reported in ppm downfield from $(CH_3)_4Si$.

ESR spectra of the spin-labeled silk fibroin membranes containing GOD were obtained at 25°C with a JEOL JES-FE3XG ESR spectrometer with an X-band. The GOD contained in the membrane was ca. 5% (w/v) and this amount of GOD was much larger than that in the GOD-immobilizing membrane prepared previously (less than 1% (w/v)) (Kuzuhara *et al.*, 1987).

Results and Discussion

Fig. 1 shows the ^{13}C NMR spectra of *B. mori* silk fibroin and a mixture of *B. mori* silk fibroin and GOD in aqueous solution. The concentration of *B. mori* silk fibroin and GOD was 6.9 and 1.35% (w/v), respectively. *B. mori* silk fibroin gave very sharp ^{13}C NMR peaks inspite of a fairly high molecular weight, $3.6\text{–}3.7\times10^5$ (Komatsu, 1982). This is due to the high mobility of the main chain, which is characterized by a very small correlation time, in the order of 10^{-10} sec for the segment motion at 40°C (Asakura *et al.*, 1984; Asakura, 1985).

In contrast to the *B. mori* silk fibroin, GOD did not give a high resolution NMR spectrum in the fibroin-GOD solution, as shown in Fig. 1b. As mentioned below, the GOD spectrum was not observed even when the concentration of GOD was 6.8% (w/v). These results are

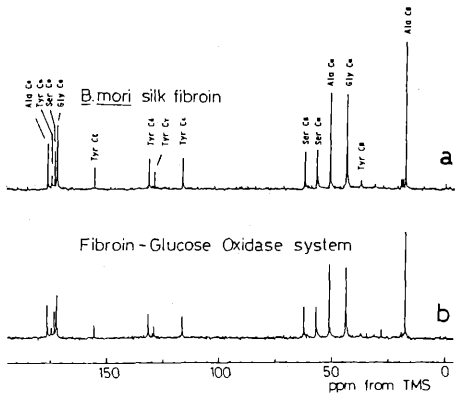

Fig. 1. ^{13}C NMR spectra of *Bombyx mori* silk fibroin (6.9% (w/v)(a) and the silk fibroin-GOD mixture (GOD concentration is 1.35% (w/v) (b) in aqueous solution.

attributed to the very low mobility of the GOD chain in the NMR time scale, indicating the maintenance of high-ordered local and whole structures even when GOD and silk fibroin coexisted. Appearance of some peaks of GOD would be expected if the local denaturation of GOD occurred as a result of a strong interaction between silk fibroin and GOD molecules. A comparison of Fig. 1a and 1b shows that the ^{13}C NMR chemical shift and curve of *B. mori* silk fibroin did not change. The GOD content in the GOD-immobilized *B. mori* silk fibroin membrane in a previous study (Kuzuhara *et al.*, 1987), was max. 1% (w/w), and the GOD content in the GOD-silk fibroin mixture in this NMR study was very high (ca. 20%(w/w)).

In order to analyze the influence of GOD on the molecular motion of *B. mori* silk fibroin, the spin-lattice relaxation times (T_1) of the protonated carbons of *B. mori* silk fibroin were observed (Table 1) for the solution with the same silk fibroin-GOD composition as that shown in Fig. 1b. The T_1 value for every carbon remained unchanged within experimental error

Table 1. ^{13}C NMR spin-lattice relaxation times (T_1) of the protonated carbons of *B. mori* silk fibroin in aqueous solution (conc. 6.9% (w/v)) at 25°C

	T_1 (sec.)	
	Fibronin only	Fibronin+GOD (a)
Gly C_α	0.086	0.087
Ala C_α	0.15	0.17
C_β	0.29	0.31
Ser C_α	0.17	0.19
C_β	0.13	0.16
Tyr C_δ	0.16	0.22
C_ε	0.15	0.21

(a) GOD concentration of this system was 1.35% (w/v).

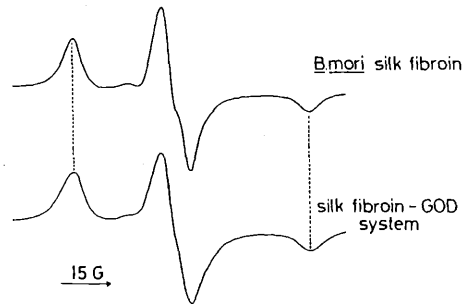

Fig. 2. ESR spectra of the spin-labeled *B. mori* silk fibroin membrane and the GOD-immobilized silk fibroin membrane (conc. ca. 5% (w/w)).

even when GOD was present. These data indicate that GOD did not affect the structure and motion of *B. mori* silk fibroin at least in an aqueous solution.

Moreover, in order to analyze the influence of GOD on the molecular motion of *B. mori* silk fibroin membranes in the solid state, the ESR spectra of the spin-labeled *B. mori* silk fibroin membranes were observed (Fig. 2). Although the GOD content was very high (ca. 5% (w/w)), the ESR spectra were still similar

even when GOD was entrapped in the membrane. These results suggest that the correlation time for the rotational motion of the nitroxide radical is longer than 10^{-8} sec (Asakura *et al.*, 1987) and is insensitive to small changes in the motion. Thus, further examination concerning the GOD-silk fibroin interaction was not possible. However, it is evident that no new mobile ESR components of the partially denaturated silk fibroin originating from the silk fibroin-GOD interaction were formed and thus that silk fibroin did not undergo denaturation in the presence of GOD.

Subsequently, the GOD-silk fibroin interaction in aqueous solution was examined in terms of GOD activity with glucose as a substrate by ^{13}C NMR spectroscopy. The ^{13}C NMR spectra of the mixture, GOD-silk fibroin-glucose, indicated the change in the electronic density distribution and motion of each ^{13}C nucleus of the 3 kinds of molecules, silk fibroin, glucose and the product gluconolactone simultaneously through the enzyme reaction as well as the rate of the enzyme reaction. If specific interactions such as silk fibroin-glucose and/or silk fibroin-gluconolactone complex formation were to occur, it would be possible to analyze the structure of the complex including the molecular site of the interaction. In addition, the relative content of α and β anomers of glucose and gluconolactone can be easily determined. The ^{13}C NMR spectra of the glucose-GOD solution system were observed immediately and 7 days after glucose was added to the GOD aqueous solution (Fig. 3). The main components observed here were the substrate glucose and the product gluconolactone, respectively. Thus, it was possible to follow the GOD reaction directly by observing the changes of the ^{13}C NMP spectra. The changing patterns in the spectra of the GOD and glucose mixture, after the GOD reaction started, are shown in Figs. 4 and 5 in the presence and absence of *B. mori* silk fibroin, respectively. The spectra were determined in about 10 min blocks. With the time course of the GOD reaction, the relative peak intensities of the carbons in glucose decreased, while those of gluconolactone increased. However, no peak broadening was observed for glucose and gluconolactone during the NMR observation irrespective of the presence of silk fibroin. Fig. 6 summarizes the time

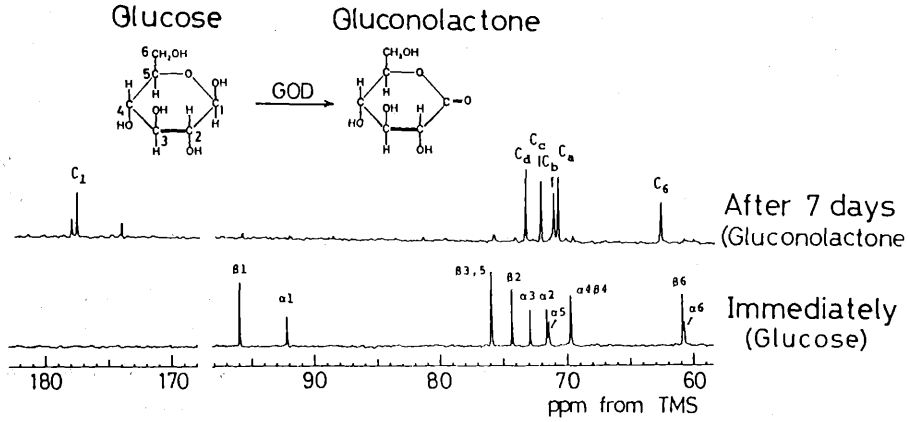

Fig. 3. ^{13}C NMR spectra of glucose-GOD system observed immediately and 7 days after glucose was added to the GOD solution. The concentrations of glucose and GOD were both 6.8% (w/v).

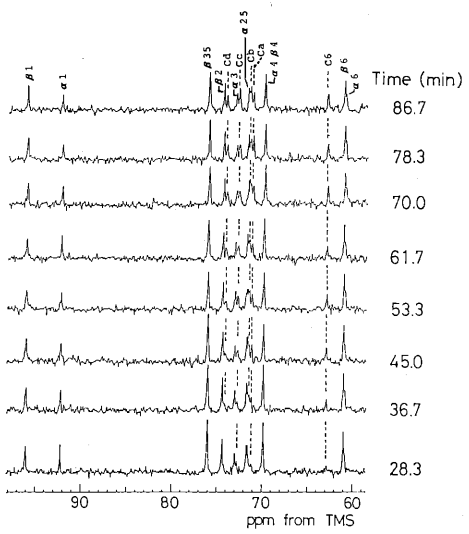

Fig. 4. ^{13}C NMR spectra of glucose-GOD system as a function of time (min) after the onset of the GOD reaction. The concentrations of glucose and GOD were the same as in Fig. 3.

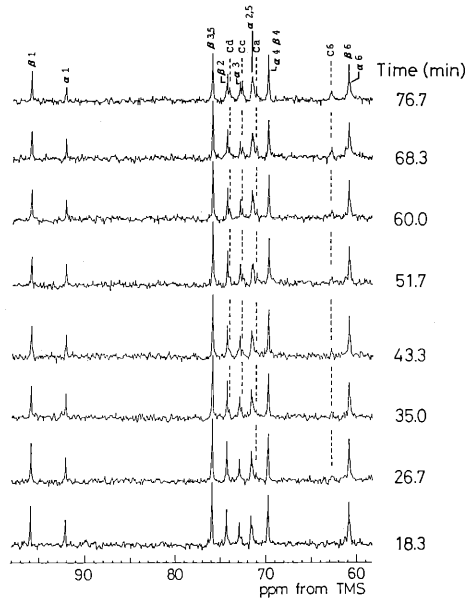

Fig. 5. ^{13}C NMR spectra of glucose-GOD system in the presence of *B. mori* silk fibroin as a function of time (min) after the onset of the GOD reaction. The concentrations of glucose and GOD were the same as in Fig. 3 and the concentration of *B. mori* silk fibroin was 2.5% (w/v).

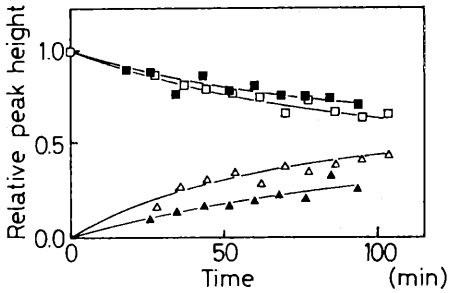

Fig. 6. Plots of the β_6 and C_6 peak heights of glucose and gluconolactone, respectively, vs. time (min) in the glucose-GOD system and glucose-GOD-*B. mori* silk fibroin system after the GOD reaction started.
Glucose-GOD system···□ : β_6, △ : C_6
Glucose-GOD-*B. mori* silk fibroin system·····■ : β_6, ▲ : C_6

dependencies in the relative peak intensity of the β_6 and C_6 carbons of glucose and gluconolactone, respectively. In the presence of *B. mori* silk fibroin, the rate of the GOD reaction slightly decreased, although no peak broadening in the spectra was observed. This phenomenon may be ascribed to a slight decrease in the diffusion of the substrate glucose and, as a result, a slight decrease in the contact probability between glucose and GOD when silk fibroin was present rather than to silk fibroin inhibition. Thus, the acceleration of the diffusion of the substrate in the silk fibroin matrix or increase in the contact probability between enzyme and substrate seems to be critical for the development of the superior enzyme-immobilizing ability of silk fibroin for an enzyme with a high activity.

In addition, the interaction between amino acids and the silk fibroin was studied using the ^{13}C and ^{1}H NMR methods. Since enzymes basically consist of 20 amino acids and in some cases amino acids are substrates, such a study becomes important when silk fibroin is widely

used as an enzyme-immobilization material. Since the carbonyl ^{13}C nuclei of amino acids and silk fibroin are relatively sensitive to the interaction compared with other ^{13}C nuclei, the changes in the carbonyl resonance region were examined in the presence or absence of silk fibroin. The concentrations of each amino acid and *B. mori* silk fibroin were 1.7 and 4% (w/v), respectively. Fig. 7 shows the carbonyl NMR spectra of *B. mori* silk fibroin containing each of 14 kinds of amino acids, together with the spectra of silk fibroin alone. The carbonyl chemical shifts of these amino acid compounds alone are also shown as stick spectra. It is worth noting that the ^{13}C NMR chemical shifts of *B. mori* silk fibroin and each amino acid hardly changed when these compounds were mixed, suggesting that there was no appreciable interaction between silk fibroin and these amino acids. For other amino acids, the interaction with silk fibroin was examined in a basic aqueous solution because the solubility of such amino acids is low in a neutral aqueous solution. However, since the spectra of silk fibroin and the amino acids in the mixture are more sensitive to small changes in pH in a basic solution than in a neutral solution, further studies were not performed. For N-acetyl-L-alanine methylamide and N-methyl-acetamide, the chemical shift and peak pattern of each amide did not change appreciably when silk fibroin was mixed. A similar experiment was performed by using ^{1}H NMR. Generally, ^{1}H NMR is more sensitive to the intermolecular interaction than ^{13}C NMR because the hydrogen atoms are located at the relatively external side of the skeleton of these molecules. However, the ^{1}H NMR spectra of silk fibroin, 20 amino acids and the 2 amide molecules were hardly altered in the binary

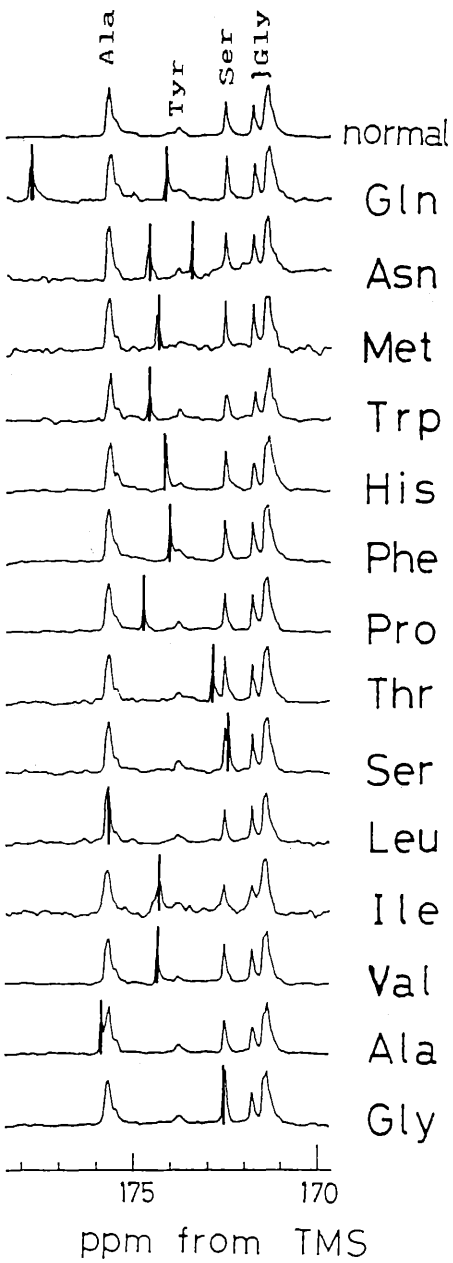

Fig. 7. ^{12}C NMR spectra of the carbonyl region of *B. mori* silk fibroin and 14 kinds of amino acids. The concentrations of each kind of amino acid and *B. mori* silk fibroon were 1.7 and 4% (w/v), respectively. The corresponding spectra of these amino acids alone were also shown by stick bars.

solution system. These results lead to the same conclusion as that reached by the ^{13}C NMR study.

It is therefore concluded that *B. mori* silk fibroin is a very convenient biomaterial for enzyme-immobilization due to the absence of a specific interaction between the enzyme and the silk fibroin molecules.

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朝倉哲郎・吉水広明・葛原亜起夫・松永　是：絹フィブロインによるグルコースオキシターゼの固定化機構

絹フィブロインが，グルコースオキシターゼ，GOD の固定化担体として優れている原因を磁気共鳴法，すなわち NMR, ESR を用いて検討した。絹フィブロインの ^{13}C-NMR スペクトルならびにスピン-格子緩和時間，スピンラベル試料の ESR スペクトルは，すべて，GOD が存在しても，変化は認められなかった。また，GOD の酵素反応は，絹フィブロインが存在しても本質的に変化しなかった。以上のように，絹フィブロインと GOD 間に目立った相互作用は認められず，絹中で GOD はその酵素反応を十分に行うことができ，それが上記の原因の一つと考えられた。