Fibrous Support for Immobilization of Enzymes

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Synopsis

A poly(vinyl alcohol) (PVA) fiber support incorporating various aminoacetal functional groups has been developed for immobilizing enzymes. The aminated PVA fiber seems to adsorb enzymes with electrostatic force of attraction; thus the immobilization procedure is simple. By the use of this fiber having immobilized enzymes, the reaction between enzymes and substrates is nearly independent of the size of substrates. This newly developed type of fiber, which is formed by a mass superfine fibers (SFF), each measuring 1 μm or less in diameter, permits much more increased surface area than the conventional enzyme immobilization supports. Our studies of the properties of the fiber for immobilization of enzymes show the following results: (1) SFF has a greater ability for the immobilization of invertase than ordinary fibers; (2) dimethyl-aminated SFF has the best performance for the immobilization of invertase. From these results, it is concluded that the dimethyl-aminated SFF is an excellent support for the immobilization of invertase.

INTRODUCTION

In the face of energy and natural resource shortages, enzymatic reactions have become of interest in recent years, because enzymes offer a number of advantages as industrial catalysts over conventional chemical catalysts such as high specificity, obviation of severe processing conditions, and high reaction rate.

Since immobilized enzymes are reusable and more stable than free enzymes, they have been widely noted and actively studied. There are some methods available for the immobilization of enzymes such as:[1,2]

(1) adsorption at a solid surface;
(2) trapping in crosslinked gels;
(3) crosslinking by bi- or multifunctional reagents;
(4) covalent bonding.

No one method seems to have a clear advantage over the others. The development of an effective method meeting various industrial requirements has been tried. We have had a hard time studying enzymes entrapped in a PVA gel,[3-6] which is able to incorporate various aminoacetal functional groups. It is difficult to operate continuous flow reactors with enzymes immobilized in the gel because flow resistance is very high for gels. An entrapment has a drawback of large diffusional barriers to the transport of a substrate and a product, and is greatly influenced by the size of a substrate. In the present work, an attempt has been made to develop the fibrous support for enzyme immobilization which adsorbs enzymes by ionic bonds. The newly developed type of fiber leads to an increase in the surface area for adsorbing enzyme. The fibrous support permits a variety of fibrous shapes suitable for intended application such as knitted fabric, string, filter paper, etc., and is expected to have low flow resistance in a column.

Journal of Applied Polymer Science, Vol. 27, 1665–1674 (1982)
 CCC 0021-8995/82/051665-10$02.00

The purpose of this investigation is twofold: (1) to see if SFF has a great ability for the immobilization of invertase; (2) to learn what kind of functional group has the best performance for immobilizing invertase.

EXPERIMENTAL

Preparation of SFF Samples

A partially saponified PVA (saponification value 80–93 mol %) and a completely saponified PVA, of which the averaged degree of polymerization ranges from 500 to 2000, are mixed in a 4:6 to 8:2 weight ratio. Poly(ethylene oxide) (PEO) was often used in the place of a partially saponified PVA. When the thread made by dry spinning of the mixture is washed, only partially saponified PVA dissolves in water. An extremely fine PVA fiber is made in this way.[7]

The following three methods[8–11] were employed to obtain aminated (see Fig. 1) and sulfonated SFF samples.

(A) PVA powder was heated at 90°C for 1 h and then was treated in the mixture of 0.15% aminoacetaldehyde dimethyl acetal and 20% H_2SO_4 at 40°C for 10 h. After washing, the powder and partially saponified PVA (or PEO) were left in hot water at 90°C for 5 h to dissolve completely. An aminated SFF was made by dry spinning of this solution.

(B) Untreated SFF was made to react with aqueous bromoacetaldehyde dimethyl acetal at 30–40°C for 2 h, and then left in 4% formaldehyde solution at 50°C for 4 h. The SFF was further treated in the mixture of 13% dimethylamine and 10% H_2SO_4 at 80°C for 20 h.

(C) After the treatment with bromoacetaldehyde dimethyl acetal and formaldehyde described in (B), the SFF was crosslinked with Na_2S and was sulfonated in the solution of 80% Na_2SO_3 at 120°C for 20 h.

All the aminated SFF samples, except that employed in the experiment described in the subsection on the influence of nitrogen content in the Results and Discussion Section, were made by the method (A).

After the SFF, aminated according to the methods described above, was left

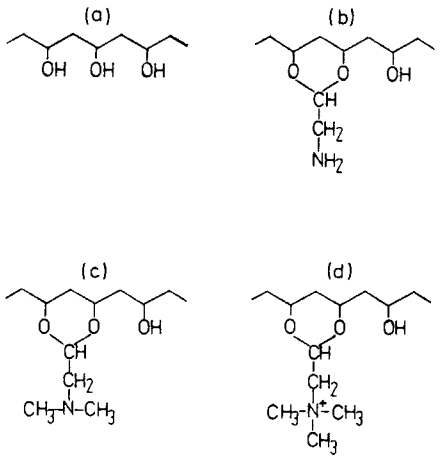

Fig. 1. Chemical structures of the aminated SFF samples: (a) original SFF; (b) aminated SFF; (c) dimethyl-aminated SFF; (d) trimethyl-aminated SFF.

in hot water at 80°C for 1 h, it was immersed overnight in 1 N HCl under agitation. For immobilization, an SFF sample was suspended in a given invertase solution under agitation at 30°C for about 20 h.

Activity Measurement

Invertase of Seikagaku-kogyo derived from *candida utilis* was used throughout this work.

Native or immobilized invertase was placed in 50 mL of 10 wt % sucrose solution adjusted to pH 4.5 by HCl and samples were withdrawn at given time intervals. [Native invertase had optimum activity at pH 4.5, but the immobilized enzyme never showed optimum above the isoelectric point. A preliminary experiment revealed that the bound invertase released from a support much more in buffer solutions, such as $(HOOC)C_6H_4(COOK)$—NaOH, KH_2PO_4, and CH_3COOH—CH_3COONa, than in water adjusted to pH 4.5 by HCl.] In the experiment to estimate the Michaelis constant K_m, sucrose solution ranging in concentration from 0.125 to 20 wt % was employed. The samples were colored by mixing with the color-producing reagent, GOD-PODLK, of Nagase-Sangyo. The amount of glucose produced is determined by measuring the absorbance of the colored solution at 505 nm with the Hitachi 101 spectrophotometer.

Pore Size Distribution

According to the method described by Urano,[12,13] the weight of methanol adsorbed on each sample was measured with the Tanaka Kagakukikiseisaku Kabushikigaisha AS-703-A instrument at varying temperatures, and then the pore size distribution and a surface area were calculated. A detailed procedure for the calculation is also described by Urano.[12]

RESULTS AND DISCUSSION

Search for the Best Functional Group for Immobilizing Invertase

The amount of invertase adsorbed on SFF samples, which are aminated and sulphonated SFF, and the immobilized enzyme activity are measured in order to learn what kind of functional group has the greatest ability for immobilizing invertase.

Table I shows the yield of adsorption and observed activity of adsorbed invertase. The yields of adsorption on all the aminated SFF samples are higher than 85%. The dimethyl-aminated SFF sample adsorbed invertase in almost a 100% yield, and the immobilized enzyme showed high activity. Using the trimethyl-aminated SFF sample, the yield is close to 90%, but the observed activity is only 6%. This might be due to conformational alteration. As to sulphonated SFF samples, the Na type of sulphonated SFF shows neither yield nor observed activity. Although the H-type sample adsorbed a lot of invertase, it shows little activity.

From these results, dimethyl-aminated SFF is found to be the best support for immobilizing invertase and was used throughout the following experiments.

TABLE I
Data from Adsorption Experiments with Various SFF Samples

Sample		Ion-exchange capacity (meq/g)	Enzyme added A (U/g)	Residual enzyme B (U/g)	Adsorbed enzyme $A - B$ (U/g)	Yield of adsorption (%)	Observed activity C (U/g)	Relative activity $C/(A - B)$ (%)
Aminated SFF		0.56	1811	0	1811	100	480	27
Methyl-aminated SFF		0.63	1850	270	1580	85	230	15
Dimethyl-aminated SFF		0.55	1750	30	1720	98	760	44
Trimethyl-aminated SFF		1.00	2400	270	2130	89	143	7
Sulphonated SFF	H type	1.1	3928	250	3678	94	226	6
	Na type	1.1	3802	3611	191	5	0	0

The Effect of a Superfine Fiber

As shown in the electron-micrographs of Figure 2, SFF is found to be much finer than an ordinary PVA fiber. Therefore, the amount of adsorbed enzymes is expected to increase with a surface area.

The partial specific volume of most enzymes is about 0.07 mL/g,[14] and the molecular weight of yeast invertase is 270,000.[15] The diameter of the invertase molecule is evaluated to be 85.5 Å by assuming that the invertase molecule is spherical in shape. It can therefore be presumed that pores larger than 85.5 Å are effective for adsorption of invertase. As to SFF, relatively large size pores form a large proportion of pore size distribution, as shown in Figure 3, where curves a, b, c and d, e represent three kinds of SFF and two kinds of charcoal, respectively. Besides, the surface area of SFF samples is as large as 10–20% of that of activated charcoal. Therefore, SFF seems to be a good support for adsorbing enzymes.

There is a great difference in the adsorption rate of invertase between SFF and an ordinary fiber (see Fig. 4). The yield of adsorption of invertase with SFF is 93% at 5 min and 97% at 240 min, while the yield with an ordinary PVA fiber is only 9% at 5 min and 26% at 240 min. As the quantity of invertase added in this experiment was small, the maximum of adsorbed invertase was not found from these results. However, it is obvious that SFF has an even greater ability for immobilizing invertase than an ordinary fiber.

The Desorption of Invertase from SFF

In general, an enzyme molecule begins to lose its negative charge and becomes detached from an ion-exchange resin as pH approaches the isoelectric point of an enzyme. As the isoelectric point of invertase from *candida utilis* is 3.8,[16] bound invertase is not considered to release from an SFF support during hydrolysis reaction. In order to ascertain this consideration, an experiment was carried out to see if adsorbed invertase detached from SFF during hydrolysis reaction at pH 4.5.

The results of the experiment are given in Figure 5. The amount of produced glucose increased linearly with time for 15 min since the beginning of the ex-

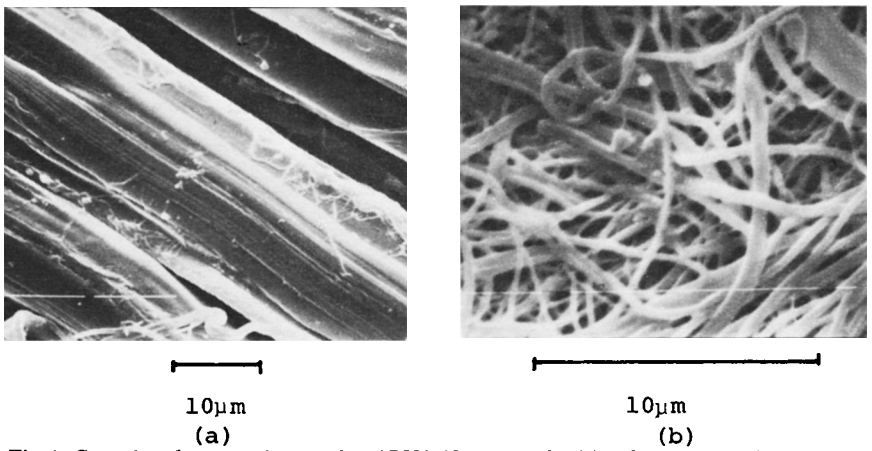

Fig. 2. Scanning electron micrographs of PVA fibers tested: (a) ordinary PVA fiber; (b) superfine fiber.

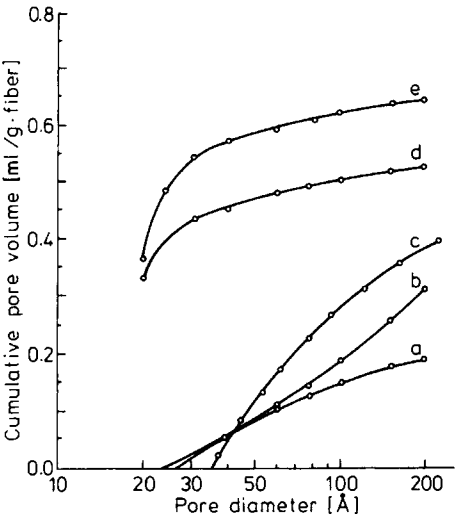

Fig. 3. Pore size distribution: (a) SFF (A; N = 0.1 wt %; S = 144 m^2/g); (b) SFF (A; N = 0.0 wt %; S = 186 m^2/g); (c) SFF (B; N = 1.1 wt %; S = 217 m^2/g); (d) activated charcoal made from petroleum pitch (KUREHA BAC-LLQ) (S = 1180 m^2/g); (e) Activated charcoal made from coconut (HOKUETSU Y-20) (S = 1400 m^2/g). A and B represent the methods of producing SFF samples described in the first subsection under Experimental. N and S represent nitrogen content in SFF and a surface area, respectively.

periment. However, it stopped increasing after the removal of the SFF-invertase conjugate. These results confirm that once adsorbed on SFF, invertase does not release from the support during the hydrolysis reaction proceeding at pH 4.5.

The Influence of Nitrogen Content on Immobilizing Invertase

Figure 6 illustrates the relationship between the total nitrogen content in SFF and the amount of invertase adsorbed on SFF. The amount of adsorbed invertase increases linearly with the nitrogen content ranging from 0.2 to 0.5 wt

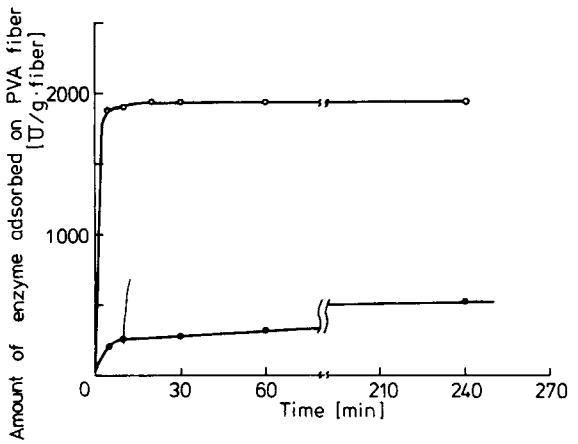

Fig. 4. Adsorption rate of enzyme by PVA fiber (N = 0.42 wt %): (—o—) SFF; (—●—) ordinary PVA fiber.

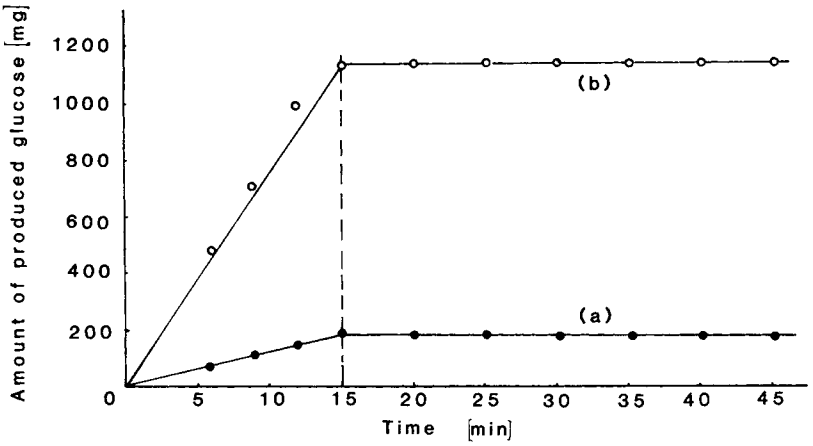

Fig. 5. Increase in the amount of produced glucose with time. The amount of invertase adsorbed on SFF (N = 0.42 wt %): (a) 1700 U/g fiber; (b) 21,000 U/g fiber.

% and reaches almost the maximum at 1.0 wt %. It seems to be reasonable that invertase is immobilized on SFF by ionic bonds. The nitrogen content of 1.0 wt % is found to be enough for immobilizing invertase.

The Determination of Michaelis Constant and Maximum Reaction Velocity

Initial reaction rates were determined at different initial sucrose concentrations ranging from 3.65 to 584 mM. Figure 7 shows Lineweaver–Burk plots for native and immobilized invertase. The values of the Michaelis constant K_m and maximum reaction velocity V_{max} for native and immobilized invertase on SFF are estimated from Figure 7 and tabulated in Table II.

The apparent K_m of the immobilized enzyme was about twice as much as that of the native enzyme. It may be due to the limitation of diffusion resistance. However, little variation of the V_{max} values suggests that the activity of immobilized invertase remains unaltered with the immobilization.

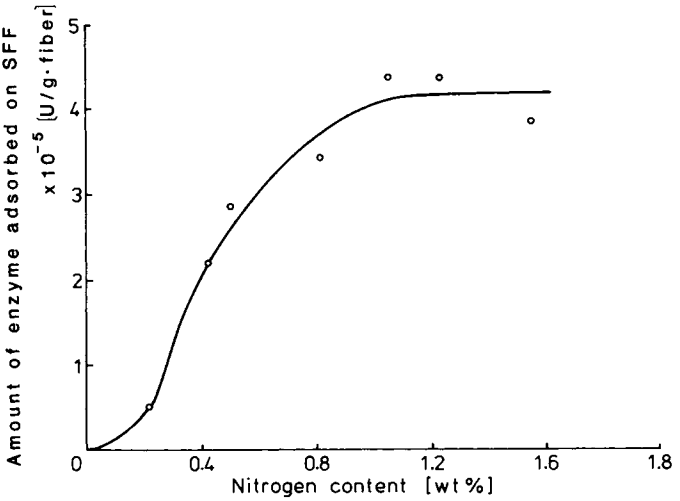

Fig. 6. Dependence of the amount of adsorbed enzyme on the nitrogen content in SFF.

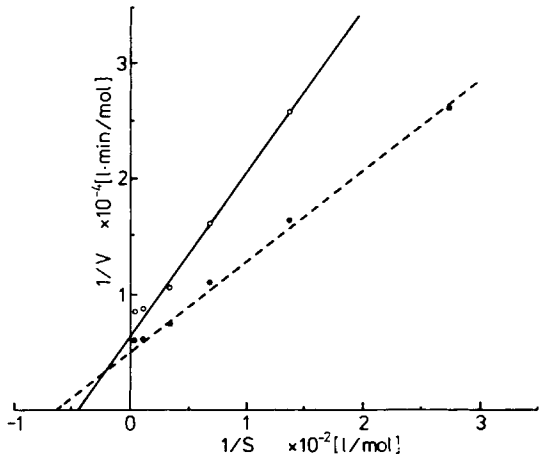

Fig. 7. Plots of $1/V$ vs. $1/S$ for native and immobilized invertase: (—●—) native; (—○—) immobilized (N = 0.42 wt %).

Dixon and Webb[17] stated that the upward deviation in the Lineweaver–Burk plot at low $1/S$ represented the high substrate inhibition. They gave the following rate equation at high substrate concentration:

$$1/V = S/(K_i \times V_{max}) + 1/V_{max} \quad (1)$$

where K_i is the inhibition constant.

Therefore, the experimental results in Figure 7 were replotted in Figure 8. Indeed reasonably good linear correlation between $1/V$ and S do exist at high substrate concentrations. However, in the case of the SFF-invertase conjugate, the slope $1/(K_i \times V_{max})$ is nearly equal to zero in the wide range of sucrose concentration, and K_i could not be determined for both the free and immobilized invertase. A finite positive value of the slope might be obtained at a much higher sucrose concentration.

TABLE II
Kinetic Parameters for Native and Immobilized Invertase

	K_m (M)	V_{max}[a] [M/min]
Native	1.18×10^{-2}	1.62×10^{-4}
Immobilized	2.57×10^{-2}	1.75×10^{-4}

[a] 1 mg of native invertase and 1.48 mg of immobilized invertase were used in this experiment.

CONCLUSIONS

The properties of the newly developed superfine fiber for the immobilization of an enzyme were studied, and the following conclusions obtained:

(1) Dimethyl-aminated SFF has the best performance for the immobilization of invertase.

(2) SFF is as fine as 0.1–0.6 μm and has great surface area. Therefore, it adsorbs a greater amount of enzyme more quickly than an ordinary PVA fiber. Moreover, it is found that an invertase does not release from an SFF support during hydrolysis reaction proceeding at pH 4.5.

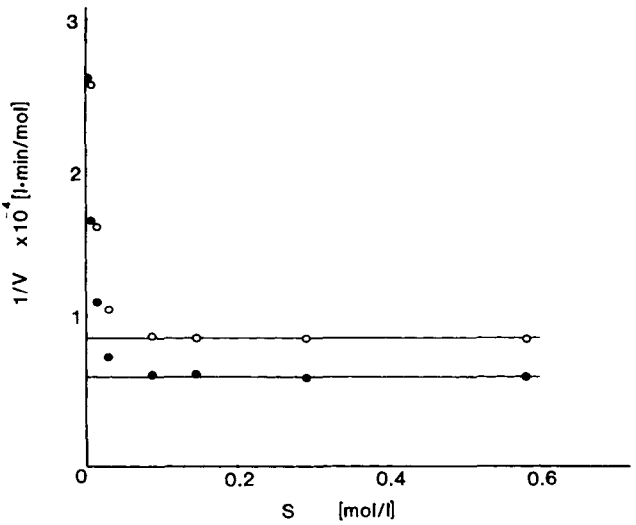

Fig. 8. Plots of $1/V$ vs. S at high sucrose concentration: (—●—) native; (—○—) immobilized (N = 0.42 wt %).

(3) An enzyme seems to be immobilized on SFF by ionic bonds because the amount of adsorbed invertase increases with the nitrogen content in SFF below 1.0 wt %.

(4) The apparent Michaelis constant K_m of the immobilized invertase is about twice as much as that of the native enzyme. The values of V_{max} are almost the same. These results suggest that there may be a limitation of diffusion resistance, but the activity of immobilized invertase remains unaltered with the immobilization.

The authors wish to express their appreciation to Dr. Junichi Nagasawa for many helpful discussions and to Miss Fumiko Sakai for her energetic assistance.

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Received July 31, 1981
Accepted October 26, 1981