Isothiocyanato-Substituted Hydrophilic Polymer Beads for Immobilization of Enzymes

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Synopsis

Styrene–glycidylmethacrylate–divinylbenzene resin beads could be converted to highly hydrophilic resin beads by acid treatment. These resin beads could be modified with isothiocyanato (NCS) groups quite easily using a Friedel–Crafts reaction and a subsequent nucleophilic reaction with KSCN. The resin beads substituted with NCS groups were useful as supports to immobilize enzymes, because they were not only hydrophilic but also bind proteins covalently. The enzymes immobilized onto the resin beads showed sufficiently high catalytic activities for a long period.

INTRODUCTION

Many kinds of enzymes have been immobilized onto solid supports for practical uses. To be suitable supports for immobilization of enzymes, solid materials must satisfy certain requirements: They have to have an adequate size and strength, and have to be easily modified with enzymes. Furthermore, the enzymes immobilized onto them should retain high catalytic activities.

The enzymes immobilized onto highly hydrophobic solid supports tend to be inactivated or denatured because of strong nonspecific interactions between those supports and the enzymes. Additionally, these supports very often show a disadvantageous adsorption of substrates and reaction products. It is, therefore, necessary to use hydrophilic materials as supports for immobilization of enzymes.[1]

For immobilization of enzymes, ion binding and physical adsorption methods are very easily carried out, but the enzymes immobilized by those methods tend to be released from solid supports gradually.[2,3] Therefore, enzymes have to be bound onto solid supports covalently to keep their activities for a long period. Additionally the covalent binding of enzymes onto solid supports has to be carried out under mild conditions to avoid the inactivation of enzymes.

Isothiocyanato (NCS) group is known to react with amino groups in proteins under mild conditions resulting in stable thiourea structures.[4] There have been only a few unsatisfactory reports about the preparation of isothiocyanato substituted polymers.[5–7] Isothiocyanato polymers so far examined were troublesome to prepare (thiophosgene was necessary)[5,6] and sometimes

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physically not so strong.[6,7] Previously we investigated a simple substitution method of NCS groups into styrene (St)–divinylbenzene (DVB) copolymer resin beads.[8] Those resin beads are, however, not highly useful as supports for immobilization of enzymes, because the resins obtained were so hydrophobic that the enzymes bound were inactivated to a large extent.

Glycidylmethacrylate (GMA) is a relatively polar monomer because of an epoxy group, and was shown to be copolymerized with styrene easily.[9] Polymer resin beads containing GMA can be obtained by using styrene and divinylbenzene as comonomers. The epoxy group which is contained in St–GMA–DVB resin beads can be converted to hydrophilic hydroxyl groups quite easily by treating with an acid solution,[10] and the obtained beads are preferable as supports to immobilize enzymes.[11]

In this report resin beads with various hydrophilicities were obtained by changing the GMA content. All of these beads could be substituted with NCS groups easily and the resin beads modified were shown to have more advantageous properties than the nonsubstituted resin beads as the supports to immobilize enzymes.

EXPERIMENTAL

Materials

Trypsin (EC3.4.21.4 from bovine pancreas 11680 unit/mg) and alkaline phosphatase (ALP, EC3.1.3.1 from calf intestine 1.4 unit/mg) were purchased from Sigma (St. Louis, MO). Substrates such as *p*-nitrophenyl phosphate disodium salt (PNPP) and *N*-tosyl-L-lysine methylester hydrochloride (TLME) were analytical reagents of Nacalai Tesque (Kyoto, Japan).

Poly(St/DVB/GMA) resin beads NEG-I–NEG-IV prepared by an suspension polymerization of styrene, divinylbenzene, and GMA [initiator 2,2′-azobis(2,4-dimethylvaleronitrile), dispersion agents; poly(methacrylic acid-co-sodium *p*-styrenesulfonic acid), and poly(vinyl alcohol)] were kindly donated by Mr. Ryosuke Nishida, Nihon Exlan Co., Okayama, Japan. The polymer beads obtained were treated with formic acid for 3 h at 90°C. Bio-Beads S-X4 were obtained from Bio-Rad. The characteristics of the resins examined are shown in Table I. All resins were washed several times with water and methanol, and dried *in vacuo*. The resin beads were passed through testing sieves to obtain particles whose diameters are in the range of 74–177 μm.

Sodium polystyrenesulfonate (NaPSS, $M_w = 2.98 \times 10^5$, degree of sulfonation = 0.67), poly-4-vinyl(*N*-ethylpyridinium) bromide (C_2–PVP) and poly-4-vinyl(*N*-benzylpyridinium) chloride (Bz-PVP) were prepared in our laboratory.

Other reagents were commercially available. Deionized water was distilled just prior to use for preparation of sample solutions.

Determination of Hydrophilicity of the Beads

The hydrophilicities of the resin beads were compared with each other from the amount of adsorption of various polyelectrolytes using a repetitive injection method by an auto-sample injector (EA-25, Kyoto Chromato, Kyoto, Japan).[12]

TABLE I
Characteristics of Resin Beads

Resins	Constitution (%) St:	GMA:	DVB	Substitution[a] (%)	Products[b]
S-X4	96	0	4	52.5	NCS
NEG-I[c]	80	10	10	34.5	NCS
NEG-II[c]	60	30	10	29.6	NCS, SCN (mixture)
NEG-III[c]	40	50	10	25.3	NCS, SCN (mixture)
NEG-IV[c]	10	80	10	20.0	NCS, SCN (mixture)

[a] Percent of substituent vs. styrene unit.
[b] NCS = isothiocyanato group; SCN = thiocyanato group.
[c] Treated with formic acid at 90°C for 3 h prior to the substitution of NCS groups.

Substitution of Isothiocyanato Groups into the Beads

Isothiocyanato resin beads were prepared by the following method: Resin beads were coupled with 3-chloropropionyl chloride by the Friedel–Crafts reaction using $AlCl_3$ as catalyst in dichloromethane at 0°C for 48 h. The resins obtained were reacted with KSCN in DMF solution at 80°C overnight.[8] The NCS group was identified by the infrared spectrum using a IR440 spectrophotometer (Shimadzu, Kyoto, Japan). The NCS content of the resin beads was determined by elemental analysis.

Immobilization of ALP

The resin beads (200 mg) degassed beforehand were suspended in 2 mL of ALP solution [2 mg/mL in pH 8.3 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer (0.05*M*)]. The suspension was incubated at 25°C for 3 h. The beads were washed with a pH 9.3 CHES buffer (0.05*M*) several times. The ALP-carrying beads (200 mg) were packed in a thermostated glass column (ID 10 × 50 mm). A solution of PNPP [dissolved in pH 9.3 CHES buffer (0.05*M*) containing 8 mM $MgCl_2$] was passed through the column at 25°C using a peristaltic pump (SJ-1211, Atto, Tokyo, Japan). The catalytic activities of ALP immobilized onto the NEG resin were determined by an increase in absorbance of *p*-nitrophenol anion at 430 nm using a U3400 spectrophotometer (Hitach, Tokyo, Japan).[8,13]

Immobilization of Trypsin

Two milliliters of trypsin solution (0.5 mg/mL) were incubated with the resin beads (200 mg) in a pH 8.0 hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (0.05*M*) buffer containing 10 m*M* $CaCl_2$ at 5°C for 1 h. The resin beads were washed several times with the same buffer solution and suspended in water. The catalytic activity of the immobilized trypsin in the hydrolysis of TLME was determined using a pH stat titrator (RTS622, Radiometer, Copenhagen).[14] The reaction temperature was maintained at 25 ± 0.05°C by a Neslab water bath RTE-8.

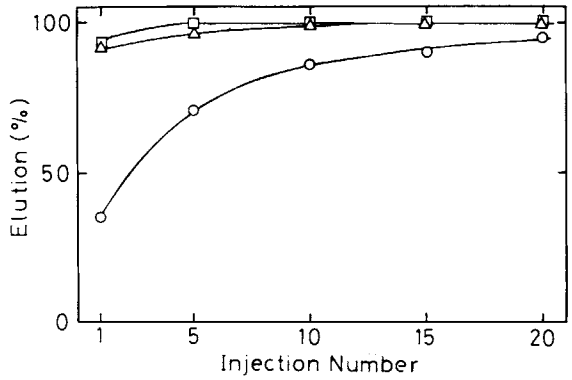

Fig. 1. Elution profiles of NaPSS from the columns packed with various resin beads at 25°C. Ten microliters of NaPSS (0.01 mg/mL) was injected each time: (○) S-X4; (△) NEG-III; (□) NEG-IV.

RESULTS AND DISCUSSION

Hydrophilicity of the Beads

At first we examined the degree of hydrophilicity of the resin beads. Figure 1 shows the elution profiles of NaPSS from the columns packed with various resin beads. The S-X4 which contained no GMA residue adsorbed the anionic polyelectrolyte, NaPSS, most strongly among all resin beads examined here. As for the NEG resins, the adsorption capacities for NaPSS decreased with an increase in the GMA content.

The NEG-IV adsorbed cationic polyelectrolytes such as C_2-PVP and Bz-PVP more strongly than other resin beads examined here. No significant difference was found in the adsorption capacity of the resin beads examined here for C_2-PVP and that for Bz-PVP (Table II).

These results suggest that an increase in the content of GMA in the resin beads increase the surface hydrophilicity. Furthermore, a part of GMA residues in the NEG beads might be hydrolyzed to methacrylic acid, which makes the NEG beads charged anionically.

Substitution of Isothiocyanato Groups into the Beads

Next, we examined substitution of NCS groups into various resin beads. Elemental analysis showed that the content of nitrogen in the resin beads was equivalent to that of sulfur, which means that NCS and/or SCN (thiocyanato

TABLE II
Amount of Cationic Polyelectrolytes Adsorbed[a]

Resins	Amount of adsorption (μg/g gel)	
	C_2-PVP	Bz-PVP
S-X4	25	24
NEG-III	230	160
NEG-IV	230	250

[a]At 25°C.

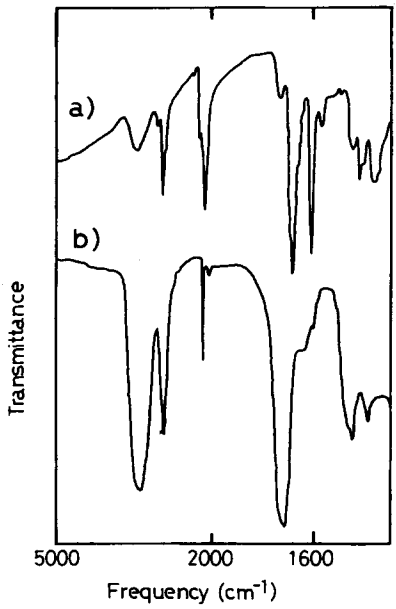

Fig. 2. Infrared spectra of NEG resins substituted with NCS (or SCN) groups: peak at 2080 cm^{-1}, NCS group; at 2160 cm^{-1}, SCN group. (a) NEG-I; (b) NEG-IV.

group) were substituted into the resin beads. The substituted groups were identified by infrared spectra.

The S-X4 and NEG-I were almost completely substituted with NCS groups [Fig. 2(a)]. The amount of SCN groups substituted increased gradually with the content of GMA in the resin beads (NEG-I, II, III). The NEG-IV beads, which contained the largest amount of GMA, were substituted with only a small amount of the NCS group [Fig. 2(b)], though the reason is not clear at this moment.

Covalent Binding of ALP to the Modified Beads

Table III shows the amount of ALP bound onto various resin beads, and reaction velocity of the hydrolysis of PNPP catalyzed by the immobilized ALP which was packed into the column. Among the parent resin beads, S-X4 bound ALP most strongly. High percentages of GMA in the resin beads seemed to decrease the amount of ALP bound, but to increase the maximum value of the reaction velocity. These results show that the highly hydrophilic supports is advantageous to immobilize enzymes.

As for nonsubstituted resin beads examined the reaction velocity by the immobilized ALP columns decreased rapidly with the continuous operation, and the operational stabilities of the immobilized ALP were almost independent of the content of GMA in the nonsubstituted resin beads. Enzymes might be easily desorbed from the nonsubstituted resin beads with a continuous operation, because they were weakly bound only by the physical adsorption and/or ion binding.

No significant change in the amount of ALP bound was observed between substituted- and non-substituted-resin beads. This is because the binding capacity of the resin beads for proteins depends on the effective surface area

TABLE III
Characteristics of Immobilized ALP Columns[a]

Resins	Amount of ALP Bound (mg/g gel)	Reaction velocity (nmol/mg min)	
		Maximum	After 100 h
S-X4	2.3	14	0.7
S-X4-NCS	2.8	20	4.2
NEG-I	1.2	19	8.8
NEG-I-NCS	1.1	170	150
NEG-II	0.7	34	7.8
NEG-II-NCS	0.2	700	550
NEG-III	0.4	130	11
NEG-III-NCS	0.3	620	560
NEG-IV	0.3	450	12
NEG-IV-NCS	0.7	240	130

[a] In 0.05M CHES buffer (pH 9.3) at 25°C. Flow rate = 0.05 mL/min.

of the beads. On the contrary, the maximum value of the reaction velocity increased by the substitution of NCS groups except the NEG-IV-NCS. This suggests that the enzymes were immobilized onto a terminal position of the long spacer ($-\phi-(C=O)-CH_2-CH_2-NH-(S=O)-$) with an advantageous conformation to carry out a catalysis.[14,15]

Only a small decrease was observed in the reaction velocity of the ALP covalently bound onto the NCS-substituted resin beads (especially NEG-III) even after an operation for 100 h (Fig. 3, Table III). From Table III the NEG-II-NCS and NEG-III-NCS beads seemed to be more favorable supports to immobilize enzymes than other resin beads examined here, probably because these two kinds of beads have a sufficient amount of binding sites as well as hydrophilic surfaces.

The apparent Michaelis constant (K_m') of the immobilized ALP which was packed into columns was determined by the Lineweaver-Burk plots at several flow rate of the substrate solution (Table IV). The value of K_m' for the immobilized ALP decreased with an increase in the flow rates, but they were

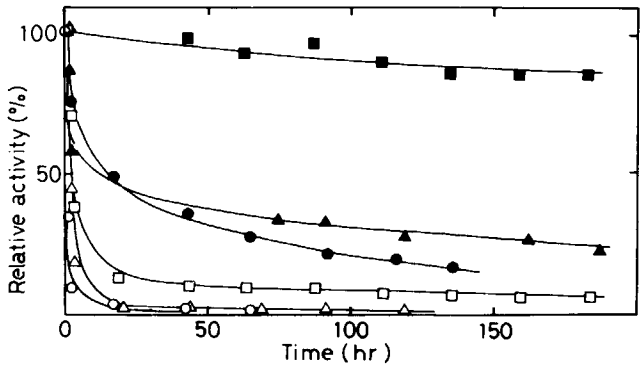

Fig. 3. Operational stabilities of alkaline phosphatase columns at 25°C, pH 9.3, 0.05*M* CHES buffer, [$MgCl_2$] = 8 m*M*, [PNPP] = 1 m*M*, flow rate = 0.05 mL/min: (○) S-X4; (△) NEG-III; (□) NEG-IV; (●) S-X4-NCS, (▲) NEG-III-NCS; (■) NEG-IV-NCS.

TABLE IV
K'_m of Immobilized ALP Columns

Resins	Flow rate (mL/min)	K'_m (μM)
NEG-II-NCS	0.05	699
	0.1	532
	0.2	374
	0.4	340
NEG-III-NCS	0.05	755
	0.1	511
	0.2	403
	0.4	287
Free[a]	—	169

[a] From batch method pH 9.3 at 25°C.

still larger than that of free ALP even at the high flow rate. These differences between K'_m of the immobilized ALP and K_m of free ALP may be due to a resistance of diffusion layer around the enzyme-carrying beads.[16]

Covalent Binding of Trypsin to Modified Beads

Next, trypsin was immobilized onto the NCS substituted resin beads. Table V shows no significant differences in the amount of trypsin bound onto various kinds of NCS-substituted resin beads, which is in contrast with the results in ALP system. This might be due to the difference of pI of ALP (pI = 4.7–5.9)[17] and that of trypsin (pI = 10.1–10.8).[18] It is well known that proteins are rapidly bound to the polymer resins mainly by the electrostatic interactions and/or hydrophobic interactions.[2] In the case of NEG resins substituted with NCS groups, the stable covalent binding between the resins and proteins is expected to occur subsequently.

By the introduction of GMA residues onto the surface of parent resins, the amount of negative charges (methacrylic acid residues) and the amount of electrically neutral hydrophilic part (glyceryl methacrylate residues) in the NCS-substituted resins might be increased. Since trypsin is positively charged in the immobilization processes (pH 8.0), it was attracted onto the surface of negatively charged NCS-substituted NEG resins (especially NEG-III–NCS and −IV–NCS). On the contrary, the amount of trypsin bound to the resins

TABLE V
Specific Activities of Immobilized Trypsins[a]

Resins	Amount of trypsin immobilized (mg/g gel)	k_2 (μmol/mg min)
S-X4-NCS	0.4	3.8
NEG-II-NCS	0.4	14.0
NEG-III-NCS	0.9	82.7
NEG-IV-NCS	0.5	34.8

[a] pH 8.0 at 25°C.

by the hydrophobic interaction might be decreased. Therefore the total amount of trypsin bound to the NCS-substituted resin beads was not largely changed by the amount of GMA residues on the surface of parent resins. The trypsin bound was less than that of ALP, because the molecular weight of a trypsin (M_w = 23,800)[19] is about 1/5 of that of ALP (M_w = 100,000).[20]

The difference of the content of GMA in the resin beads affected the reaction rate constant of enzyme-substrate complex (k_2) of the immobilized trypsin largely. The largest k_2 was obtained in the NEG-III–NCS, which shows that the NEG-III–NCS has not only the NCS group to bind enzymes covalently but also sufficient hydrophilicity to make a microenvironment around enzymes "comfortable." Trypsin bound onto the NEG–NCS beads was stable and the activity retained 60% of the initial value after the storage at 10°C in water for 1 month.

The results obtained here show that the NCS-substituted hydrophilic resin beads are very useful as the supports to immobilize enzymes.

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