Application of Porous Microspheres Prepared by SPG (Shirasu Porous Glass) Emulsification as Immobilizing Carriers of Glucoamylase (GluA)

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ABSTRACT: Fairly uniform spheres of crosslinked polystyrene (PS) and polymethyl methacrylate (PMMA), prepared by a particular emulsification process using SPG (Shirasu Porous Glass) membranes and subsequent suspension polymerization, were applied for immobilizing carriers of Glucoamylase (GluA). A mixture of monomers, solvents, and oil-soluble initiator was allowed to permeate through the micropores of SPG, suspended in an aqueous solution of poly(vinyl alcohol), and polymerized while retaining the narrow size distribution during polymerization. A small amount of acrylic acid or glycidyl methacrylate (GMA) was incorporated for the immobilization of GluA via covalent bonding. Although GluA has been regarded as being difficult to retain its activity after the immobilization process, a porous structure of the carriers definitely favored the immobilization, and a maximum 55% relative activity (RA) was obtained by the physical adsorption to PMMA spheres. The reaction of epoxide in GMA with 6-aminocaproic acid provided a spacer arm for the carboxyl group. An improvement of activity was expected by the incorporation of the spacer arms; however, barely noticeable activity was observed for PMMA carriers either by the physical adsorption or by the covalent bonding. A slight improvement was observed for PS carriers with spacers compared to the carriers without them. The diffusion process of oligosaccharides in the porous carriers seemed to retard the rate of hydrolysis in the case of largest carriers, 60 μm PS-DVB-AA spheres. The activity of immobilized GluA was retained during a long storage period of more than 150 days, some of them even increasing gradually, while the activity of native GluA dropped to zero after 100 days. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **65:** 2655-2664, 1997

Key words: SPG membrane; porous microspheres; glucoamylase; immobilization; suspension polymerization

INTRODUCTION

A new technique of preparing relatively monodisperse polymeric microspheres, the coefficient of variation (CV) being close to 10%, and the size ranging from 2 to 100 μm, has been developed in our laboratory.[1-5] A hollow cylindrical glass membrane, Shirasu porous glass (SPG), having a narrow pore size distribution, provides fairly uniform monomer droplets when a certain volume of monomer-solvent mixture, dissolving an oil-soluble initiator, and a water-insoluble reagent is allowed to permeate through the micropores. These droplets are suspended in an aqueous solution of poly(vinyl alcohol) (PVA) with a small amount of sodium lauryl sulfate (SLS), and polymerized under nitrogen atmosphere with gentle stirring. No breakup or coalescence of the droplets takes

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place during the polymerization. Addition of water-insoluble hexadecane (HD) or long-chain alcohols significantly retards the degradative diffusion process, which induces the coalescence process.[6] Controlling the size distribution in a narrow range is another essential factor to maintain the stability during the polymerization. Addition of water-soluble inhibitors such as hydroquinone[1] and sodium nitrite[2] prevents the secondary nucleation of smaller polymer particles in the aqueous phase, which plays a role as binder between the larger droplets.

The strong hydrophilic nature of SPG (composed of Al_2O_3—SiO_2) favors the formation of uniform PS particles. The average diameter of the droplets has been found to be directly proportional to the average pore size of the membrane, the proportionality constant being close to 6.[1] More hydrophilic methyl methacrylate (MMA) tends to yield a wider size dispersion because of the wetting surface of SPG. This problem has been overcome by adopting the swelling technique,[7] in which the primary hydrophobic droplets, mainly composed of benzene and HD, were swollen with the secondary emulsion droplets of MMA and other hydrophilic components. Subsequent polymerization yielded PMMA spheres, their CV being in a comparable range as those of PS spheres.[2] Application of the swelling technique to the hydrophobic monomers provided larger monodisperse spheres as large as 100 μm.[5]

Crosslinking agents, divinylbenzene (DVB) for PS and ethyleneglycol dimethacrylate (EGDMA) for PMMA, and adequate diluents, alkanes, and middle-chain alcohols, are crucial additives to obtain porous spheres, which are regarded as sophisticated materials because of their large surface-to-volume ratio and functionality. A successful application of PS–DVB spheres as a packing material for the gel permeation chromatography (GPC) has been briefly reported.[3]

Applications as a carrier of enzyme immobilization, using polymeric microspheres having the above-mentioned size range, have been reported with only biologically favored spheres such as poly(*g*-methyl-L-glutamate) for papain,[8] PVA spheres for lipoprotein lipase,[9] tannin spheres for α-amylase,[10] and chitosan beads for papain, ficin, and bromelein.[11] These spheres all have in common porous structure and high relative activity of the immobilized enzymes.

Various attempts have been reported concerning the immobilization of Glucoamylase. Cabral and Kennedy[12] summarized the carriers employed in connection with the methods of immobilization, which include activated carbons, corn stover (lignocellulosic macroporous material), metallic oxides, porous glass, innovated cellulose, and gelatin. Cho et al.[13,14] extensively pretreated activated carbons with various acids and other chemicals, investigated the difference of morphology, H_2O_2 decomposition activity by the carbon carriers, and the hydrolytic activity by immobilized GluA. Vallat et al.[15] used hard fraction of crashed corn stover as carriers—0.3 and 0.8 mm in size, and external surface area of 318.3 and 88.5 m^2/g, respectively—and applied these for fluidized bed operations to obtain glucose from malt-dextrin, and collected engineering data useful for industrial scale-up purpose. Within our reference survey, however, no attempts have been reported dealing with granular or spherical carriers for the immobilization of GluA. Uniform spherical particles, preferably from tens to hundreds of micrometers in size, can be potential supports for industrial bioreactors and column separations.

In the previous article, the authors used micron-size NAD PS spheres as the immobilizing carrier of GluA, and proposed that the highly crosslinked porous spheres were favored to retain a high activity of immobilized glucoamylase (Im-GluA), although the relative activity was by no means in a satisfactory range.[16] Now that we have established the technique to prepare the required porous spheres, either hydrophilic or hydrophobic, this article demonstrates the versatility of these spheres. Performance of Im-GluA and the various attempts to improve the activity of GluA after the immobilization will be reported in this article.

EXPERIMENTAL

Materials

Polymer particles

Polymer particles used as immobilization carriers are summarized in Table I. Detailed descriptions of the preparation have been given elsewhere.[1–5] All the PMMA spheres and PS-2448 were obtained by using the swelling process. After the polymerization, polymer particles were washed with methyl alcohol (Kishida Chemical Co.) under sonification to remove solvents and unreacted monomers, washed again with methyl alcohol, and distilled water, and finally dispersed in the distilled water and stored. A SEM photograph of 2448 is shown in Figure 1. The coarse surface

Table I Polymer Particles Used as Carriers for Glucoamylase

Carrier No.	Monomer	d_p (μm)	CV (%)	A (m^2/g)	[—COOH] (U/nm^2)
402	ST-DVB-AA (0.2)[a]	7.63	15.6	54.9	0.15
411	ST-DVB-AA (0.2)	3.62	14.1	73.6	0.36
412	ST-DVB-AA (0.2)	5.14	36.2	108	0.13
416	ST-DVB-AA (0.2)	5.20	10.9	142	0.10
427	ST-AA (0.2)	6.60	14.8	1.91	14.7
2448	ST-DVB-AA (6.3)	60.1	14.9	39.3	0.20
606	ST-DVB-GMA (0.9)	8.24	11.2	138	0.36[b]
380	MMA-EGDMA-AA (0.2)	4.99	8.18	138	0.070
386	MMA-EGDMA-AA (0.2)	37.3	13.4	185	0.090
601	MMA-EGDMA-AA (1.9)	17.7	15.4	115	0.047
381	MMA-AA (0.2)	4.75	9.10	1.26	6.95
920	MMA-EGDMA-GMA (1.0)	15.8	11.0	74.4	—
922	MMA-EGDMA-GMA (1.0)	15.1	13.3	41.6	0.051[b]

[a] Weight percent of acrylic acid or GMA to the total weight of monomer.
[b] —COOH was incorporated by the ring-opening reaction of epoxides in GMA with 6-aminocaproic acid.

suggests larger pore size, and corresponds to a small specific surface area of 39.3 m^2/g.

Enzymes

Glucoamylase (Gluczeim AF6, Rhizopus delemar, Amano Pharmaceutical Co.) was a food-processing grade containing a trace amount of α-amylase and water-insoluble extenders. Unidentified extenders were removed by filtration from the aqueous suspension prior to immobilization.

α-Amylase (Bacillus sp., Sigma) was used to hydrolyze soluble starch (Kishida) to prepare this substrate.

Reagents

1-Ethyl-3-(dimethylaminopropyl) carbodiimide (carbodiimide activator, Sigma) and 6-aminocaproic acid (spacer, Kishida) were used. Disodium hydrogen phosphate, anhydrous, and potassium dihydrogen phosphate (Kishida) were used for the preparation of buffer solutions (pH = 5.5, 7.0, and 8.0). Polyoxyethylene nonylphenylether (POE-NPE, Kao Co.), with 23 OE units, was used to investigate the effect of adsorbed surfactant on the activity of Im-GluA.

Potassium hydroxide (0.01*M,* Kishida) was used for the conduct metric titration for measuring carboxyl groups on the surface of carriers, and controlling pH in the reaction mixture.

Dyestuff Brilliant Blue G (Coomassie Brilliant Blue G, approx. 60 wt % purity, Sigma) was used to determine the percent of immobilization of the enzyme. Phosphoric acid (85%, Kishida) was also used. All these reagents were biochemical or reagent grade, and used as received.

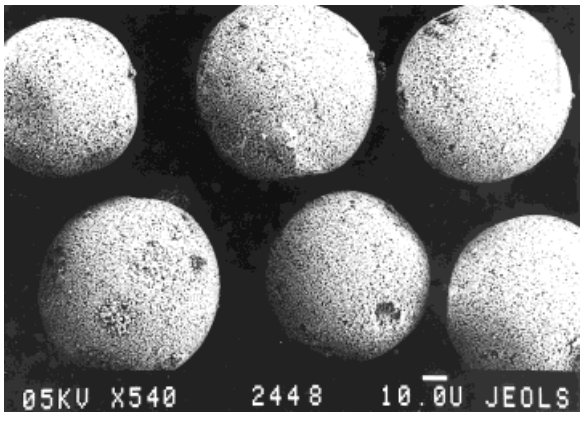

Figure 1 SEM photograph of PS-2448. d_p = 60.1 μm, CV = 19.9%, A = 39.3 m^2/g.

Immobilization of Glucoamylase

As the detailed description of the immobilization process via covalent bonding was given elsewhere,[17] only a comprehensive diagram including the adsorption process is shown in Figure 2.

Incorporation of Spacer Arms

Polymer particles incorporating GMA, and 6-aminocaproic acid were added to distilled water of pH over 9.0 and allowed to stand at 313 K for 6 h under mild agitation. Polymer particles were washed several times with distilled water after the reaction and stored suspended in distilled water.

Evaluation of the Activity of Im-GluA

Soluble starch (10 g) was dissolved in 1 dm^3 of distilled water, and treated with Am at 310 K for 24 h. pH of the filtered solution was adjusted to 5.0, and served as the oligosaccharide solution, the concentration of which was 10 g/dm^3.

When the activity of free GluA was measured, 0.63 mg of the enzyme was added in 100 mL of the oligosaccharide solution, and the hydrolysis was carried out at 313 K until nearly 100% yield of glucose was attained. A certain amount of polymer particles equivalent to 1.3–1.4 mg of Im-GluA was added in 100 mL of the substrate solution, when the activity of Im-GluA was measured. The rest of the procedures were as same as those for free GluA.

Three different lots of GluA were used throughout this work, and the initial activity was expressed as the rate of glucose formation, r_H (mg-Glu/mg-GluA/min), was plotted against the substrate concentration in Figure 3. The rate was obtained by graphical differentiation of the Glu yield versus time curve employing the mirror image method.[18] No distinct difference in the activity of free GluA was observed except for a few scattered points corresponding to the later stage of hydrolysis. In ordinary experiments, r_H was calculated from a slope of the straight line drawn from the initial part of glucose yield versus the time curve. As indicated in the figure, 26.5 mg-Glu/mg-GluA/min was selected as the average r_H of free GluA when the relative activity (RA) of Im-GluA is discussed. RA was defined as follows:

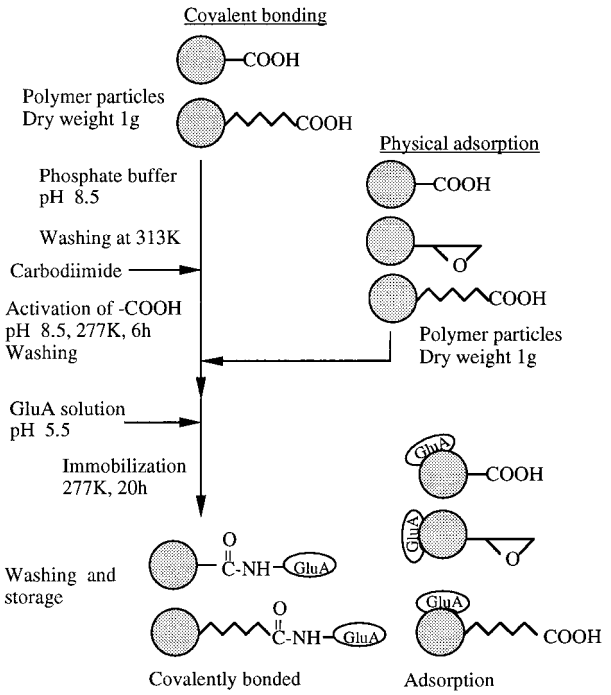

Figure 2 Schematic diagrams of two immobilization processes.

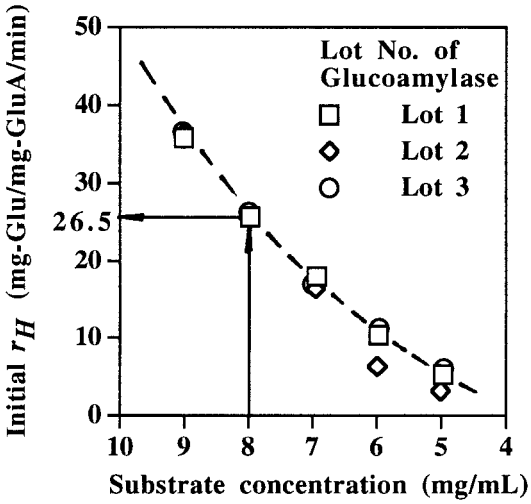

Figure 3 Initial activity of free Glycoamylase expressed as the rate of glucose formation, r_H, against substrate concentration.

$$\mathrm{RA} = \frac{\text{Average } r_H \text{ of Im-GluA (mg-Glu/mg-Im-GluA/min)}}{26.5\ (\text{mg-Glu/mg-GluA/min})} \times 100(\%) \quad (1)$$

The surface coverage defined below is sometimes used for the correlation of r_H.

$$\phi = \frac{(\text{Surface area occupied by Im-GluA})}{(\text{Total specific surface area of solid latex})} \times 100$$

$$= \frac{(\text{Number of Im-GluA molecules}) \times a_G \times (10^{-18})}{(\text{Total specific surface area of solid latex})} \times 100(\%) \quad (2)$$

where a_G is the coverage area of a GluA molecule, and assumed to be $5.1 \times 5.0 = 25.5\ nm^2$.[19] Molecular weight of GluA was assumed to be 58,000 Daltons.[20]

Analyses

After the immobilization, the amount of free GluA remaining in the serum was determined by the

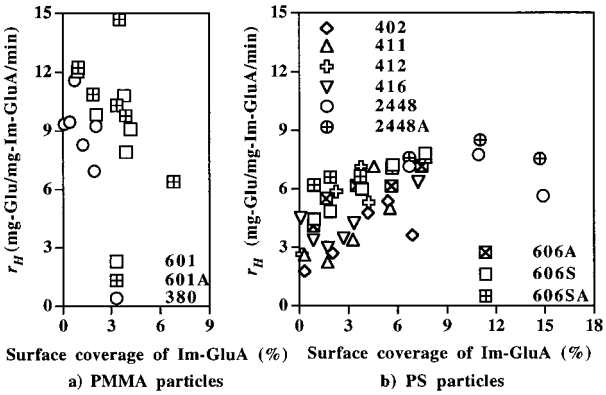

Figure 4 Activity of Im-GluA, r_H, as a function of the surface coverage. Suffix A in the data No. indicates immobilization by adsorption. Suffix S in the data No. indicates the incorporation of a spacer.

Coomassie Brilliant Blue (CBB) method as proteins.[17]

The concentrations of oligosaccharides composed of up to 10 glucoside units were determined by using a HPLC apparatus (HPLC-8010, Toso Co.), the column was packed with polyamide gel (TOSOH Amide-80), and the detector was a refractive index meter (TOSOH RI-8010).

The details of the analytical procedure have been given elsewhere.[16]

RESULTS AND DISCUSSION

Effect of Structure of Polymer Particles

As shown in Table I, two carriers, PS-427 and PMMA-381, have no porous structure, thereby no appreciable surface area. Barely noticeable activity of GluA remained after the immobilization with these carriers, although an appreciable percentage of Im-GluA was observed. It is obvious that the large specific surface area is one of the primary characteristics polymer particles have to possess as enzyme carriers.

General Performance of Im-GluA

The activity of GluA immobilized on various sizes and features of polymer particles was plotted against the surface coverage of Im-GluA, and is shown in Figure 4.

Hydrophilic spheres such as PMMA–EGDMA have been regarded less favorably than the hydrophobic PS spheres as a material for protein manipulations. However, except for PMMA-386 (only 8% RA at maximum), r_H maintained higher values compared to PST carriers, the highest value being 14.7 mg-Glu/mg-Im-GluA/min or 55% RA. The data points scatter in the range of lower surface coverage, partly due to the lower incorporation of acrylic acid (—COOH) as shown in Table I, and probably due to the less favorable surface for the adsorption of GluA, as shown later.

As for the hydrophobic PS carriers, r_H increases as the surface coverage becomes higher, attains a plateau at around 10% surface coverage, and starts to decrease from 15% thereafter.

This tendency suggests the possible limitation of the substrate molecules diffusing through the pores to reach active sites of GluA immobilized near the center of spheres, in particular, run 2448.

Site of Immobilization

As shown in Figure 1 and elsewhere,[5] the porous structure develops uniformly throughout the carrier particles even though the size of the particles becomes bigger. Whether free GluA is able to penetrate through the pore to the very center of sphere, and is immobilized uniformly, has to be discussed before tackling the diffusion of substrate species.

Carlaysmith et al.[21] measured the penetration rate of proteins into a porous support by staining support beads and splitting to expose the cross-section. Penicillin acylase and bovine serum albumin (BSA) tagged by fluorescein were allowed to penetrate into Amberlite XAD-7. The following theoretical solution was derived to estimate the time required for the diffusional front to reach the center of the particle:

$$t = \frac{q d_p^2}{24 D_e C_0} \tag{3}$$

where q and C_0 are the concentration of the penetrant in the porous support and in the bulk solution, respectively; d_p is the diameter of the particle; and D_e is effective diffusion coefficient in the porous support. They proposed D_e to be in the range of $1-5(10^{-12})$ m^2/s. Equation (3) and the value of D_e may be applied to our system because GluA having a dimension of $5.2 \times 5.0 \times 5.0$ nm^3 [19] diffuses through the pores of approximately 100 nm of diameter.[1] Substituting $q/C_0 = 1$, $dp = 60(10^{-6})$ m, and $D_e = 10^{-12}$ m^2/s into eq. (3) will yield

$$t = \frac{[60(10^{-6})]^2}{(24)(1)(10^{-12})} = 150 \text{ s} \tag{4}$$

Because this value is by far smaller than the actual immobilization time of 20 h, or the activation period by carbodiimide reagent of 6 h, it will be a reasonable assumption that GluA is immobilized uniformly in the support spheres.

Limitation of Substrate Diffusion to the Catalysis

In order to investigate a possibility that the hydrolysis of oligosaccharides is retarded by the diffusional resistance of the substrates, the commonest way is to compare Michaelis constant, K_m, of immobilized enzyme obtained from the Lineweaver–Burk plot with that of free enzyme. Normally, K_m of the former becomes higher depending on the extent of diffusion resistance.

In the case of GluA, however, Nakamura et al.[20] and Kusunoki et al.[22] claimed that the hydrolysis of low molecular weight saccharides is retarded by the competitive inhibition of the product, glucose, although Vallat et al.[23] found a slight increase of $v_{\max}$ in their corn stover-supported GluA by using high molecular weight maltodextrins as a mixed substrate. In our system, the Lineweaver–Burk plot failed to yield a straight line, r_H decreasing more rapidly with diminishing concentration of the substrate than those Michaelis–Menten mechanism predicts. The next most probable way is to compare time scales of the diffusion and the hydrolysis. As there is no external diffusion resistance between the bulk solution and the particles due to sufficient agitation applied to the suspension, only the time scale of internal diffusion should be considered by using eq. (3). The diffusion coefficient of maltose in ion exchange resins (DoweX 50W-X8, H^+ type) or in activated carbon particles is reported to have an order of $1–2.4(10^{-10})$ m^2/s.[24] Substituting this value in eq. (3), and again assuming $q/C_0 = 1$, we obtain

$$t_D = \frac{[60(10^{-6})]^2}{(24)(1)(10^{-10})} = 1.50 \text{ s} \qquad (5)$$

On the other hand, the time scale of the hydrolysis can be estimated by using the free GluA data shown in Figure 3, and Figure 5 to be shown later, which provide the data on immobilized GluA/g-support. Assume the porosity of carrier 2448 to be 0.5, and the density to be unity, then 1 g of the support is equivalent to 1 mL of pore volume. All the experiments were carried out with the initial concentration of the substrate, 1 g/100 mL, which is equivalent to 10 mg/mL. Picking 20 mg-GluA/g-support as the value of im-GluA on the support, the rate of Glu formation was 50 mg-Glu/mg-GluA/min from the respective figures, and RA was 0.3. From these data, the time scale of the hydrolysis can be estimated as

$$t_H = \frac{10(\text{mg} - \text{oligosaccharide/g} - \text{support})}{0.3(50)(\text{mg} - \text{Glu/mg} - \text{GluA/min})(20) \times (\text{mg} - \text{GluA/g} - \text{support}) \times (60)(\text{s/min})} = 2.00 \text{ s} \qquad (6)$$

provided that the weight of oligosaccharide and Glu are equivalent, and can be canceled in eq. (6).

Comparison between t_D and t_H may lead to a false conclusion that the catalysis proceeds a bit slower than the diffusion process; however, this is not the case. In the above arithmetic, the rate of hydrolysis was evaluated as pretty low because the true rate should be defined with respect to the total numbers of cleaved 1,4-glucoside linkage, which is by far larger than r_H. As a conclusion of this section, it is apparent that the limitation of the diffusion process is mainly responsible for the declining activity of Im-GluA with increasing surface coverage. The diffusion of the substrates through the pores of support particles controls the apparent rate of Glu formation. Nonetheless, deactivation of GluA during the immobilization process is another major factor for the decrease of r_H. Notice that the time scale of diffusion is proportional to the square of particle size, that is, the diffusion in the carrier 380 (d_p = 4.99 mm) should be more than 100 times faster than 2448. However, maximum RA remained as 55% in spite of this advantage.

Obviously, the higher surface coverage is desirable for commercial applications on account of the efficient utilization of carriers. Vallat et al.[15] employed very high ratios of GluA(g)/support(g) for their immobilization process, 16/30 and 6.5/200 for corn stover particles of 0.2 and 0.8 mm, respectively. Despite such high loadings of GluA, 10–15% RA was retained. An advantage of the corn stover supports is their high external surface area(macro) compared to the inner one(micro), the former being 88.5(0.8 mm) to 318.3(0.2 mm) m^2/g and the latter a mere 0.6 m^2/g. In run 412, higher GluA feed ratios to the carrier, 100 and 200 mg-GluA/g-carrier, were tried. The resulting RA were only 9.1 and 2.5%, respectively, and excluded from the plots in Figure 4. More intensive designing of the pore size of the carrier particles will be a worthwhile target to pursue as our future experiments.

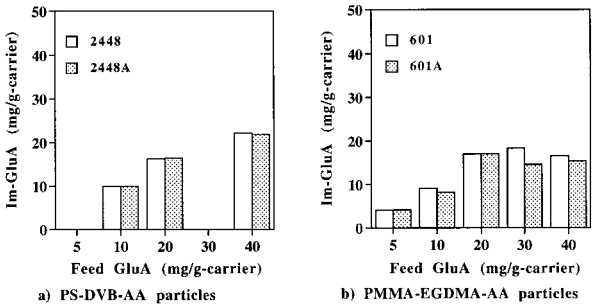

Figure 5 Amount of Im-GluA against that of feed GluA, difference between covalent and adsorbed immobilization. Hydrophobic PS and hydrophilic PMMA spheres are compared.

Adsorption of GluA on the Carrier Particles

The amount of Im-GluA, either by covalent bonding or by physical adsorption, is plotted against the feed GluA in Figure 5, and the corresponding activity, expressed as the rate of glucose yield r_H, is plotted against the same abscissa in Figure 6. As mentioned already, PS-2448 is the biggest sphere with a coarse porous structure, while PMMA-601 reveals a smaller pore. An interesting result is that the amount of immobilized GluA is not dependent on the different mode of immobilization regardless of the type of carrier particles. The amount of Im-GluA increased linearly with the feed GluA for PST-2448. On the other hand, it reached a saturation plateau for PMMA-601, the plateau value being less than 20 mg/g-carrier. The adsorption behaviors of PS(hydrophobic) and PMMA(hydrophilic) carriers agreed well with the experimental fact that the hydrophobic surface offers better adsorption sites than the hydrophilic one.

From Figure 6 the activity of GluA immobilized by adsorption indicates slightly higher values than those by covalent bonding. Combining the results shown in Figures 5 and 6, it can be assumed that the GluA molecules dissolved in the suspension medium at first adsorb on the surface of carrier particles, and then undergo reaction to form peptide bonds in the case of immobilization by covalent bonding.

The interaction between the adsorbed GluA and the carrier surface seems to be strong, because washing the suspension several times with distilled water did not yield a noticeable amount of desorbed GluA. The experimental evidence that there is no big difference in activity between the two immobilization processes may indicate that the inner structure of the enzyme molecules is probably perturbed during the attachment to the surface and rearrangement.[25]

Incorporation of Spacer Arms on the Carrier Particles

Incorporation of spacer arms, in other words, stretching out the functional group (—COOH in our case) from the carrier surface, has been accepted as an effective technique to preserve the original activity of enzymes immobilized covalently. As the bonding is formed at the tip of a stretched arm, the enzyme molecule is not affected by the surface, being less affected by hydrophobic interaction, electrostatic effect by charged groups, or by attached ions, and so on. Furthermore, the number of bonds formed per one enzyme molecule will be undoubtedly reduced, even though there is no guarantee of a single bond per each enzyme molecule. Multiple bondings will distort the higher structure of the enzyme molecule, eventually reducing its activity. Hayashi and Ikada used spacer-incorporated chitosan beads (Fuji Fabric Co.), and the immobilized papain retained more than 50% of RA.[11] Also, 84% of RA was obtained with papain connected to the spacer on the surface of BMLG beads.[8]

In order to achieve the same improvements, spacer arms were incorporated on PS-606 and PMMA-922 via ring-opening of epoxides in GMA with 6-aminocaproic acid. In a moderate temperature, 313 K, epoxides preferentially react with amines, leaving free carboxyl groups at the other ends.

By using these spheres with spacers, the amount of Im-GluA, either by covalent bonding or by adsorption, was plotted against the feed GluA in Figure 7, and the corresponding activity, r_H, was plotted against the same abscissa in Figure 8. The suffixes S and SA indicate the data of covalent bonding and adsorption, respectively. Adsorption

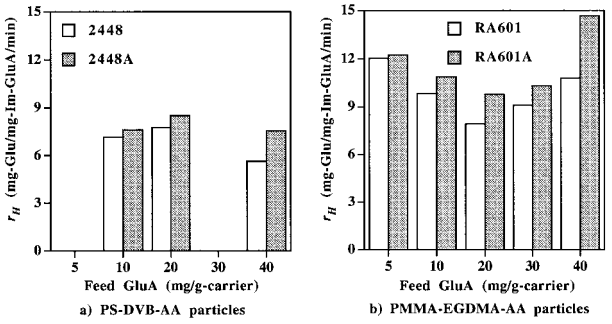

Figure 6 Activity of Im-GluA, r_H. Each column corresponds to the one shown in Figure 5.

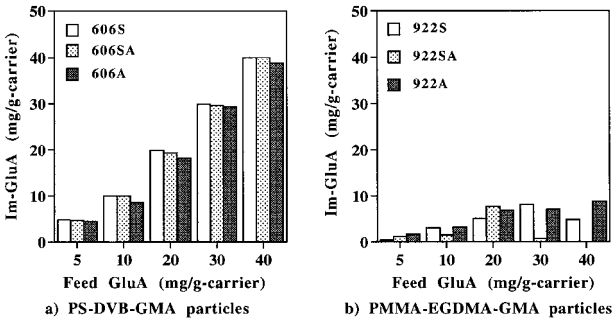

Figure 7 Amount of Im-GluA against that of feed GluA, effect of spacer incorporation. Hydrophobic PS and hydrophilic PMMA spheres are compared.

data (with the suffix A) using original PS-606 and PMMA-922 (before incorporation of the spacer) were also shown in the figures for comparison sake. There is a marked difference between the data observed with the PS and the PMMA spheres compared with those shown in Figure 6. While nearly 100% of immobilization was attained with PS-606, regardless of the incorporation of spacers and the process of immobilization, less than 10% of immobilization and poorly correlated data were obtained with PMMA-922. It should be pointed out that the higher capacity of immobilization of PS-606 (Fig. 7) compared to PS-2448 (Fig. 5) is due to the larger specific surface area, 138 to 39.3m^2/g (see Table I).

The poor activity shown in Figure 8(b) directly reflects the low immobilization results in Figure 7(b). Although not shown in detail, the other carriers, PMMA-920, revealed the same results—poor immobilization and low activity. Considering that the ring-opening of epoxides takes place to some extent during the polymerization,[26] the surface of PMMA–EGDMA–GMA spheres becomes more hydrophilic due to the formation of hydroxyl groups and unfavorable for the adsorption of GluA. Granted that the reaction with 6-aminocaproic acid progressed as expected, the hydroxyl groups are formed as well. This may be a probable explanation that even PMMA-922A (no spacer) revealed a poor performance compared to 601A.

On the other hand, the amount of Im-GluA and the resulting activity improved for hydrophobic PS-606 as shown in Figures 7(a) and 8(a). The differences in the amount of Im-GluA are not as noticeable in Figure 7(a), but the incorporation of the spacer proved advantageous to the activity. Except for the data on the low feed GluA, r_H of 606S and SA are higher than those of 606A. The improvement may not be as significant because the benefit of spacer arms will be more enhanced for GluA molecules immobilized on the external surface of the spheres. The activity of those immobilized in the pores will still be limited by the diffusion resistance of the substrates, as discussed before. Therefore, the increase in r_H of 606S and 606SA is mainly due to the favorable readjustment of the hydrophobic surface, which promotes the adsorption of GluA. In the case of immobilized papain,[11] higher RA was only obtained with the longer spacer arms formed by two peptide bonds from one hexamethylene diamine and two succinic acids.

Effect of Adsorbed Surfactant on the Carrier Surface

An investigation of the effect of adsorbed surfactant on the carrier surface is interesting in two aspects; one is the modification of the surface feature of carrier particles, and the other is to find out the possibility of using surfactants as stabilizing agents of carrier particles. For example, when a surfactant is used as a stabilizer for the further modification of carrier particles such as the incorporation of spacer, an exhaustive process of surfactant removal will be required if the presence of the surfactant causes a negative effect on the performance of immobilized enzyme.

Polyoxyethylene nonylphenylether (POE-NPE) (0.3 g) was added to the suspension containing 1 g of carrier particles, and allowed to stand prior to the adsorption of GluA. The amount of adsorbed GluA and the resulting r_H are shown in Table II, together with the standard data obtained without the addition of the surfactant. Sharp decreases in the percent immobilization and further declines of r_H obviously indicate that no advantage is expected by the use of nonionic sufactants in the immobilization of glucoamylase. The decrease of immobilized GluA does not directly lead to the nearly complete loss of the activ-

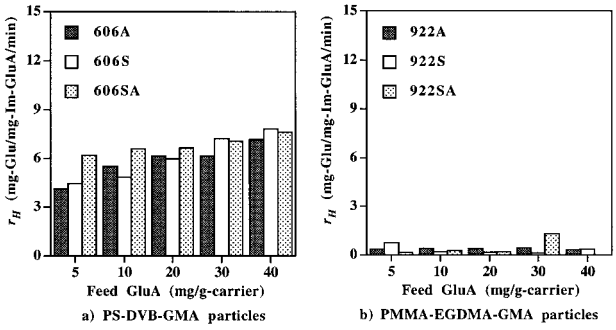

Figure 8 Activity of Im-GluA, r_H. Each column corresponds to the one shown in Figure 7.

Table II Effect of the Adsorption of Surfactant on the Im-GluA and RA

Run No.	Monomer	Feed GluA (mg/g-carrier)	Percent Immobilization	Rate of Glu Yield r_H (mg-GluA/mg-Im-GluA/min)
2448A-3	ST-DVB-AA	40.0	54.6	7.55
2448EA-3[a]		40.0	33.8	0.084
601A-1	MMA-EGDMA-AA	5.0	83.3	12.2
601EA-1		5.0	31.2	0.079
601A-5		40.0	38.5	14.7
601EA-5		40.0	19.6	0.069

POE nonylphenylether was used as a surfactant.
[a] Surfactant was adsorbed on the surface of polymer particles prior the addition of GluA.

ity. As Norde pointed out, not only is the surface transformed to protein resistant, but also the rearrangement of the secondary structure of enzyme molecules involving the deformation of α-helixes and ß-sheets takes place after the adsorption.[25]

Shelf Life of Im-GluA

It has already bben reported that Im-GluA retained its initial activity after 180 days, quite a few samples gradually going up to as high as 150% of original r_H.[3] We speculated that this gradual increase of r_H is due to a slow decay or hydration of the pore wall of the carrier particles, obviously exposing GluA to the substrate molecules, which will be captured by the active sites of GluA with less obstruction in the diffusion path. Possibly, a favorable structural rearrangement of GluA may also contribute. Native GluA, on the other hand, completely lost its activity after 100 days.[3]

In this article, the difference of longevity between covalently immobilized GluA and adsorbed GluA is shown in Figure 9. As was expected, the adsorbed GluA was less stable than that covalently bonded; however, the difference was very slight for PS-2448. Instability of the adsorbed GluA became clear when the hydrophilic PMMA-601 was used as a carrier. Although only one group of data, measured after 53 days, are shown in Figure 9, all the residual activities of the adsorbed GluA dropped while those by the covalently immobilized GluA remained intact. The result also implies that the hydrophobic porous spheres are more consistent than the hydrophilic ones as materials for immobilizing proteins.

CONCLUSION

Fairly monodisperse polymeric microspheres, either hydrophobic or hydrophilic, and the average diameters ranging from 5 to 60 μm, were proved to be potential carriers for immobilizing glucoamylase, and probably for general proteins. Porous structure, and therefore, large specific surface area, are absolutely necessary for GluA to retain its activity hydrolyzing oligosaccharides to glucose. Controlling of the pore size becomes singularly important because the diffusion limitation of substrate molecules becomes a more serious issue for larger spheres, which are favored for industrial purposes than smaller ones. Obviously, covalent immobilization is preferred; however, physically adsorbed GluA also retains a respectable longevity. Generally, PMMA carriers retained higher relative activity than PS; however,

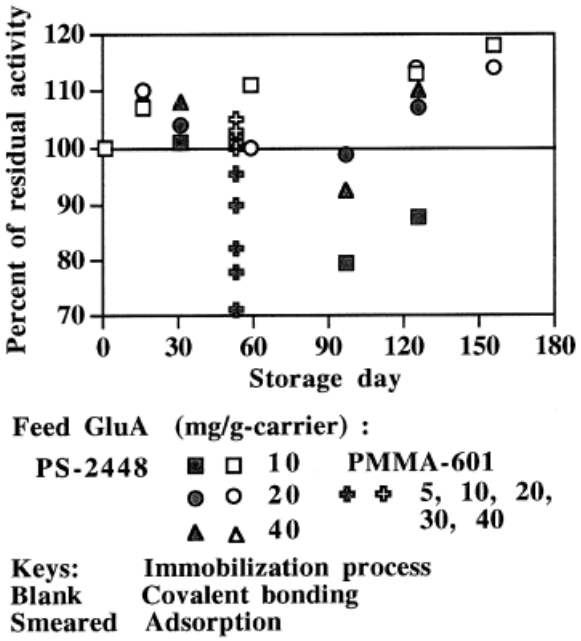

Figure 9 Residual activity of Im-GluA against storage day. PMMA data with various feed GluA are plotted with one symbol because all the measurements were only done unanimously after 53 days.

the surface coverage of GluA is by no means satisfactory for commercial applications. A delicate balancing of hydrophilic and hydrophobic ratio of PS spheres seems to be required for an optimal designing of the carrier surface. An ultimate designing concept will emerge from these studies.

Incorporating GMA in the carrier particles, and the introduction of spacer arms by the ring-opening reaction of the epoxides with 6-aminocaproic acid did not prove a clear advantage of the spacers. There is a strong possibility that the epoxide rings are opened during the polymerization at the temperature, 348 K. Whether the laborious incorporation processes of spacers are really worthwhile to try should be determined by balancing higher activity and sustainability against risking the stability of the carrier particles in a dispersion.

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