Phosphorylcholine (PC)-bonded Protein A Affinity Chromatographic Medium for High-Throughput Purification with Reduced Non-specific Protein Adsorption

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Abstract

A protein A affinity chromatographic medium based on porous silica modified with phosphorylcholine (PC) groups and amino groups (PNSP) was synthesized. The PC groups functioned as suppressors of non-specific protein adsorption. Recombinant protein A was bound to the amino groups on PNSP with a glutaraldehyde used as a spacer (PNSP-PA). The PC groups and amino groups were immobilized on porous-silica particles using two silane coupling reagents, PC-bound silane, and 3-aminopropyltrimethoxysilane. After optimizing various factors in the synthetic process, the resultant protein A medium showed improvements in non-specific protein adsorption, dynamic binding capacity, and chemical stability under basic conditions compared with conventional protein A affinity media.

Introduction

Monoclonal antibodies are important biological pharmaceuticals and are used to treat several types of cancer (1–5). Protein A affinity chromatography is commonly used for antibody purification, due to its selectivity towards antibodies in complex solutions, such as cell-culture fluids (6–8). Due to the increasing demand for monoclonal antibodies for therapeutic uses, improvements in protein A affinity media are required, especially in terms of sample throughput and recovery (9,10). Protein A affinity media based on a variety of support materials, such as cross-linked agarose, synthetic polymers, porous-glass, and porous-silica, have been studied from versatile aspects (11–19).

Cross-linked agarose is a support commonly used in protein A affinity chromatography because of its hydrophilic nature, which causes less non-specific adsorption to biological substances. Although it demonstrates a high stability in most aqueous buffers commonly used in protein A affinity chromatography, cross-linked agarose is known to show slow separation kinetics and low resistance against pressure and so may not be suitable for applications requiring a high sample throughput (at a high flow rate).

On the contrary, porous silica and glass, two of the most commonly used supports, are suited to high-flow-rate operations because of their fast separation kinetics and high mechanical strength, although they tend to non-specifically adsorb to proteins, which usually results in a reduction of product purity or recovery (9).

In this study, affinity media based on porous silica modified with phosphorylcholine (PC) groups and amino groups (PNSP) were synthesized. PC groups are expected to function as a suppressor of non-specific protein adsorption due to their high biocompatibility and hydrophilicity (20–22). Recombinant protein A (rprotein A) was bound to the amino groups on PNSP with glutaraldehyde (PNSP-PA), a spacer.

One obvious advantage of PNSP-PA is its compatibility with high-throughput purification at a high flow rate due to the nature of the silica. Another is the biological inertness of PC groups, one of the major moieties forming phospholipids in cell membranes. PC groups are known to have high biocompatibility and an electrically neutral and highly hydrophilic betaine structure (20–24). These properties cause materials modified with PC groups to show minimal non-specific protein adsorption (21,22). Furthermore, the effect of the suppression of non-specific protein adsorption by PC groups is higher than that by other general hydrophilic groups (25,26). The performance of PNSP-PA in terms of capacity of antibodies and level of non-specific protein adsorption, in comparison with those of conventional protein A affinity media, are discussed.

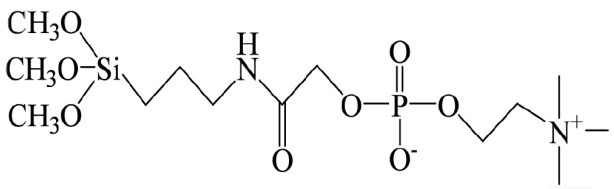

Figure 1. The structure of PC-bound silane (PCS).

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Experimental

Preparation of porous-silica particles modified with both PC groups and amino groups (PNSP)

High-purity porous silica (particle diameter of 20 µm, Shiseido, Tokyo, Japan) was used as a starting material for PNSP. For comparison, porous silica modified with only amino groups (NSP) (i.e., with no PC groups, was also prepared). The pore sizes of the porous silica used here are shown in Table I. PC-bound silane (PCS, Shiseido, Tokyo, Japan) and 3-aminopropyltrimethoxysilane (APTMOS, Wako, Osaka, Japan) were used as surface modifiers for the porous silica. The structure of PCS is shown in Figure 1. PCS and APTMOS were dissolved in water–methanol (5:95, v/v) to adjust the total volume to 400 mL (the concentration of the total silane coupling reagents: 30 mM). Several types of solutions containing different molar ratios of PCS and APTMOS were prepared as shown in Table I. Porous silica (60 g) was dispersed in each solution. Each mixture was stirred at 64°C for 5 h. The formed PNSP was separated and washed with 1 L of water–methanol (50:50, v/v), and then dried in vacuo at 80°C for 16 h.

Determination of PC groups on PNSP

Five mg of PNSP were dispersed in 1 mL of perchloric acid solution (60%, Nakarai Tesque, Kyoto, Japan). The dispersion was kept at 180°C for 1.5 h to completely degrade any PC groups present into phosphate. Then, 8.2 mg of Hexaammonium Heptamolybdate Tetrahydrate and 8.0 mg of Ascorbic acid were added to the sample solution and boiled at 100°C for 5 min. The amount of phosphate was determined by the absorbance at 710 nm (27).

Analysis of carbon contents in PNSP and NSP

The total carbon contents in PNSP and NSP were measured with an Organic Elemental Analyzer 2400CHN (Perkin-Elmer Japan, Yokohama, Japan).

Evaluation of non-specific adsorption of albumin, IgG, and lysozyme on silica, NSP, and PNSP (9:1, 100).

The non-specific adsorptions of silica, NSP, and PNSP-PA (9:1, 100) were evaluated using three different proteins with different isoelectric points, albumin (bovine, pI = 4.9), IgG (white egg, 5.0 < pI < 9.5), and lysozyme (white egg, pI = 11.0). These three proteins were purchased from Sigma-Aldrich (St. Louis, MO). Each protein solution (0.1 mg/mL with PBS) was applied to 25 mg of each particle. The mixture was stirred at room temperature for 1 h. After that, the resultant mixture was centrifuged, and 0.1 mL of supernatant was mixed with 0.1 mL of micro BCA reagent (Thermo Scientific, Rockford, IL) and boiled at 60°C for 1 h. The amounts of proteins in the supernatants were quantified by the absorbance of the solutions at 562 nm, and the amounts of adsorbed proteins on the particles were calculated by the amounts of proteins left in the supernatant.

Table I. Synthetic Results for the PNSP and NSP Used in this Study

	Pore Diameter (nm)	Surface area (m2/g)	Molar ratio		Total Carbon (%)	PC groups (µmol/g)
			PCS	APTOMS		
PNSP (9:1, 100)	100	24	0.9	0.1	0.47	14.3
PNSP (5:5, 100)	100	24	0.5	0.5	0.47	10.5
PNSP (3:7, 100)	100	24	0.3	0.7	0.47	8.8
PNSP (2:8, 100)	100	24	0.2	0.8	0.47	8.0
PNSP (1:9, 100)	100	24	0.1	0.9	0.42	4.6
NSP	100	24	0.0	1.0	0.72	–
PNSP (1:9, 70)	70	42	0.1	0.9	0.79	7.2
PNSP (1:9, 50)	50	50	0.1	0.9	0.95	8.9

Synthesis of PNSP-PA and NSP-PA (Immobilization of recombinant protein A on PNSP and NSP)

For this experiment, 0.8 g of SCBH was dissolved in 20 mL of PB and mixed with 4 g of PNSP or NSP. The mixture was stirred at room temperature for 5 min. Then, 20 mL of glutaraldehyde solution were added and the mixture was stirred at room temperature for 5 h. After removing the supernatant by centrifugation, the particles were washed five times with 40 mL of PBS. Then, the supernatant was removed again by centrifugation, and 64 mg of recombinant protein A [rprotein A, M_r (molecular weight) = 55 000, Oriental Yeast, Tokyo, Japan] and 64 mg of SCBH were dissolved in 40 mL of PB and added to the particles (28). The resultant mixture was stirred at room temperature for 16 h. After removing the supernatant by centrifugation, the particles were washed with 40 mL of PBS. Then, the supernatant was removed again by centrifugation, and 0.8 g of SCBH was dissolved in 40 mL of glycine-HCl solution (0.2 M, pH 2.5) and added to the particles for the purpose of blocking unreacted aldehyde groups. The resultant mixture was stirred at room temperature for 2 h. After removing the supernatant by centrifugation, the particles were washed five times with 40 mL of PBS.

Packing

PNSP-PA, NSP-PA, and conventional protein A affinity media were packed into Tricorn 5/100 glass tubes (100 mm × 5 mm i.d., GE Healthcare Bio-Sciences, Tokyo, Japan). The conventional protein A affinity media used were MabSelect SuRe (a cross-linked-agarose-based medium, GE Healthcare Bio-Sciences) and ProSep-vA Ultra (a porous-glass-based medium, Millipore, Billerica, MA).

Dynamic binding capacity

Dynamic binding capacity (DBC) describes the amount of IgG that binds to a gel packed in a column run under defined conditions. The DBC for any media is highly dependent on the running conditions, sample preparation, and even the origin of the sample. In general, the lower the flow rate, the higher the DBC. As the flow rate approaches zero, the dynamic capacity approaches the available capacity. DBC is determined by loading an IgG solution containing a known concentration of IgG, and monitoring IgG in the column flow-through while applying IgG solution.

Similarly to previous reports (11), DBC was defined here as the amount of IgG bound under a certain flow condition until 5% breakthrough of unbound IgG occurs. The 5% breakthrough was defined as the point at which the UV absorbance signal

reaches 5% of the maximum intensity obtained with IgG solution without a column. The structure of rprotein A immobilized on PNSP is similar to that of native protein A, and rprotein A has an affinity for human IgG (28). Therefore, the general binding conditions for protein A affinity chromatography were used. Human IgG (MP Biomedicals, Irvine, CA) was used for the determination of DBC. The IgG solution (0.5 mg/mL in 50 mM borate / 3 M sodium chloride, pH 7.0) was first applied to the system without the column in order to obtain the maximum intensity value. Then, the column was attached, and the solution was applied to the column. The flow was stopped once the UV absorbance reached 5% of the maximum in order to calculate DBC (g of IgG/L of medium).

Evaluation of the amounts of non-specific adsorption of human serum albumin (HSA) on PNSP-PA and NSP-PA

Human serum (Millipore) was used for the evaluation of the amounts of non-specifically adsorbed HSA on PNSP-PA and NSP-PA. Anti(HSA) mouse IgG (AbD Serotec, Kidlington, Oxford, UK), anti(HSA) mouse IgG conjugated with HRP (Bethyl Laboratories, Montgomery, TX), and casein (from milk, Wako) were used for the sandwich enzyme-linked immunosorbent assay (ELISA) in order to quantify the non-specifically adsorbed HSA. The method was as follows: inject human serum solution (diluted 5 times with PBS) for 9 min, wash for 10 min with PBS, elute for 4 min with glycine-HCl solution (0.2 M, pH 2.5). The flow rate (linear velocity) was 450 cm/h for all steps. The amount of adsorbed HSA in the eluted fraction was determined by sandwich ELISA. For the ELISA, anti(HSA) mouse IgG was immobilized on micro titer plate wells. Dilutions of the samples were incubated in the wells, before being incubated with anti(HSA) mouse IgG conjugated with HRP. The HRP enzymatic activity was quantified with *o*-phenylenediamine.

Chemical stability in 100 cycles of operation with 6 M guanidine-HCl

Human serum was used for the evaluation of chemical stability in 100 cycles. The method was as follows: inject human serum solution (diluted 5 times with PBS) for 10 min, wash for 10 min with PBS, elute for 4 min with glycine-HCl solution (0.2 M, pH 2.5), and equilibrate for 10 min with PBS. This process was repeated for 100 cycles. Cleaning with 6 M guanidine-HCl solution for 3 min was performed every 10 cycles. Furthermore, PBS was loaded for 3 min after 50 cycles and 100 cycles in order to quantify non-specifically adsorbed substances such as proteins, DNA, and lipids derived from human serum. The flow rate was 450 cm/h for all steps.

The IgG recovered in the eluted fraction was quantified by UV absorbance at 280 nm. Ninety-five microliters of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), 5 µL of 2-mercaptoethnol, and 100 µL of the eluted fraction were mixed and heated in boiling water for 3 min. The resultant mixture was applied to a 5–15% acrylamide gel (Bio-Rad Laboratories), and SDS-PAGE was performed. The gel was stained with Coomassie Brilliant Blue (Bio-Rad Laboratories), and the purity of the eluted fraction was confirmed. The amount of non-specifically adsorbed substances eluted in PBS was measured by a TOC apparatus (TOC-5000, Shimazu, Kyoto, Japan).

Chemical stability in 100 cycles of operation with 0.1 M sodium hydroxide

The purpose of the experiment was to evaluate the chemical stability in a basic solution during washing. However, not only the washing solution but also the sample solution seemed to be major factors that affected on stability of the column. Therefore, a small amount of IgG was selected as a sample.

The method was as follows: inject human IgG (0.5 mg/mL in PBS, pH 7.4) for 1.7 min, wash for 13.3 min with PBS, elute for 6.6 min with glycine-HCl solution (0.2 M, pH 2.5), clean for 3.4 min with 0.1 M sodium hydroxide, and equilibrate for 13.3 min with PBS (9). This process was repeated for 100 cycles. The flow rate was 450 cm/h for all steps. The peak shapes in 100 cycles were evaluated.

Results

Characterization of the PNSP

The PC groups in PNSP were intended to function as suppressors of non-specific protein adsorption, and the amino groups of PNSP and rprotein A were linked together with glutaraldehyde, a bifunctional spacer. Total carbon (C%) and the amounts of PC groups (µmol/g) in various PNSP synthesized in the study are listed in Table I. PNSP with a pore diameter of 100 nm showed an increase in the amount of PC groups dependent on the increase in the molar ratio of PCS, while total carbon contents did not change among these PNSP. On the other hand, the amounts of PC and amino groups increased in relation to the order of PNSP (1:9, 100), PNSP (1:9, 70), and PNSP (1:9, 50).

Evaluation of the amounts of non-specific adsorption of albumin, IgG, and lysozyme on silica, NSP, and PNSP (9:1, 100).

Figure 2 shows the adsorption levels of albumin, IgG, and lysozyme. Unmodified silica nonspecifically adsorbed all three proteins, especially IgG and lysozyme. By contrast, in the case of NSP, the adsorption levels of albumin and IgG were higher than that of lysozyme. On the other hand, the adsorption levels of all three proteins on PNSP (9:1, 100) were significantly low.

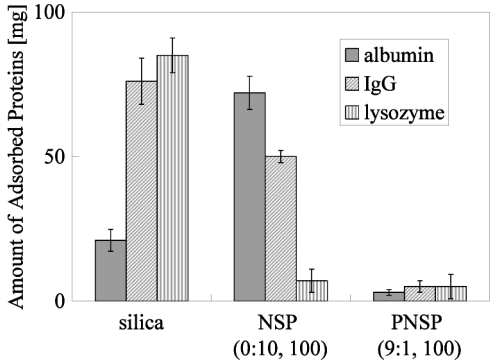

Figure 2. Amounts of non-specific adsorption of albumin, IgG, and lysozyme.

DBC of PNSP-PA

The DBC values of PNSP-PA with a pore diameter of 100 nm are shown in Figure 3. DBC values increased as the amount of PC groups decreased.

In contrast, a comparison of PNSP-PA with different pore sizes, the DBC values of PNSP-PA (PCS–APTMOS, 1:9) with pore sizes of 100, 70, and 50 nm were 37, 48, and 22 mg/mL gel, respectively. PNSP (1:9, 70)-PA showed the largest DBC value among the three.

Evaluation of amounts of non-specifically adsorbed HSA

The non-specifically adsorbed HSA values of six PNSP-PA (100 nm) with different molar ratios of PC and amino groups were compared (Figure 4). Although the amounts of adsorbed HSA in the 9:1 to 1:9 types were almost the same, the 1:9 and 0:10 types showed a difference of one order of magnitude.

Comparing the DBC of PNSP-PA with those of conventional protein A affinity media

The influence of flow rate on the DBC observed in three media, PNSP-PA, MabSelect SuRe, and ProSep-vA Ultra, is indicated in Figure 5. Flow rate had the largest influence in the case of MabSelect SuRe, whose DBC drastically decreased with flow rate. In contrast, both ProSep-vA Ultra (based on porous glass) and PNSP-PA (based on silica) were less affected by flow rate changes, and the latter had the highest DBC at all the flow rates tested.

Chemical stability in 100 cycles of operation under 6 M guanidine-HCl

To study the chemical stability of the media, the amounts of IgG recovered were measured during 100 cycles of operation. All media demonstrated consistent yields over 100 cycles, with no indication of deterioration (Figure 6). It seems common in protein A affinity chromatography that 50% of the binding capacity is loaded onto the column, and 20 mg/mL gel corresponded to that amount. The yield of PNSP-PA was the highest in all media. SDS-PAGE was performed every 10 cycles, and equivalent SDS-PAGE profiles were obtained from all the purified fractions (data not shown). Therefore, only the SDS-PAGE profile of the first purified fraction in Figure 7 is shown. All three affinity media showed two main bands, which were identified as the IgG heavy chains (about 50 kDa) and light chains (about 25 kDa). As far as purity was concerned, no clear qualitative difference was observed among the three media.

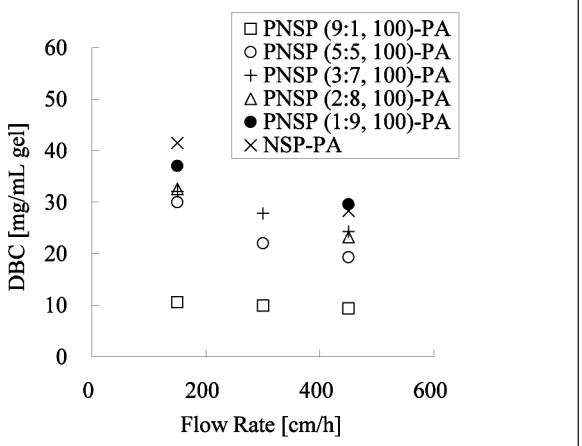

Figure 3. DBC of PNSP-PA with a pore diameter of 100 nm and NSP-PA measured on a column (100 mm × 5 mm i.d.).

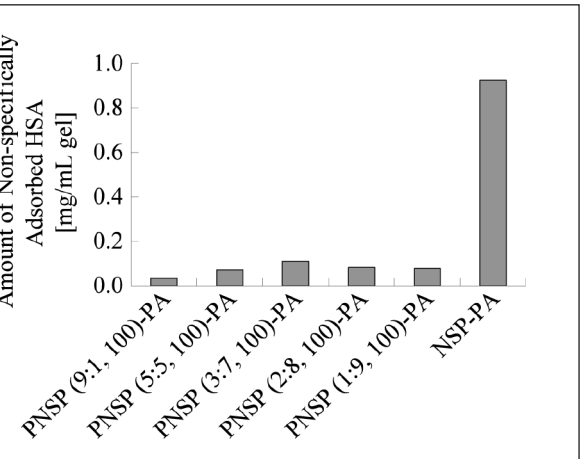

Figure 4. Amount of non-specifically adsorbed HSA quantified by ELISA.

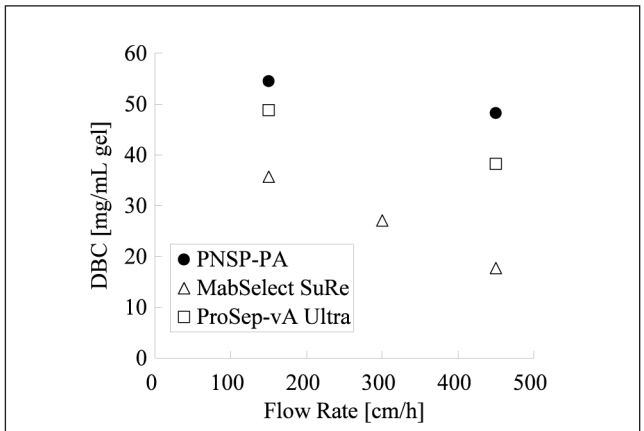

Figure 5. Influence of flow rate on the DBC of three media, PNSP-PA, MabSelect SuRe, and ProSep-vA Ultra. DBC was measured on a column (100 mm × 5 mm i.d.).

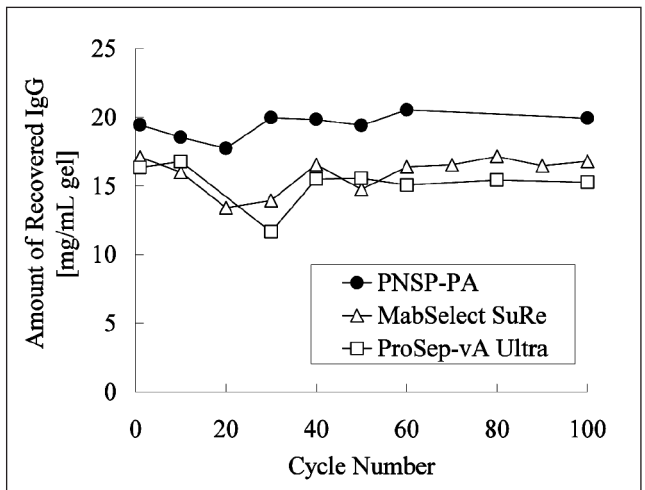

Figure 6. Amount of recovered IgG during 100 cycles of operation.

Figure 8 shows the total amounts of carbon (TOC) in the PBS fraction. TOC can be correlated to the total amount of organic compounds in general. The TOC value of PNSP-PA was extremely small in comparison with those of the conventional protein A affinity media.

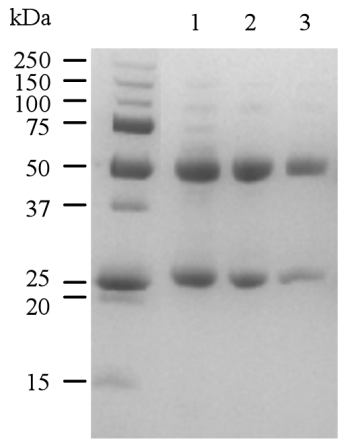

Figure 7. SDS-PAGE of IgG fractions produced by the initial cycle for each medium. The gel was stained with Coomassie Brilliant Blue. Lane 1: PNSP-PA, Lane 2: MabSelect SuRe, and Lane 3: ProSep-vA Ultra.

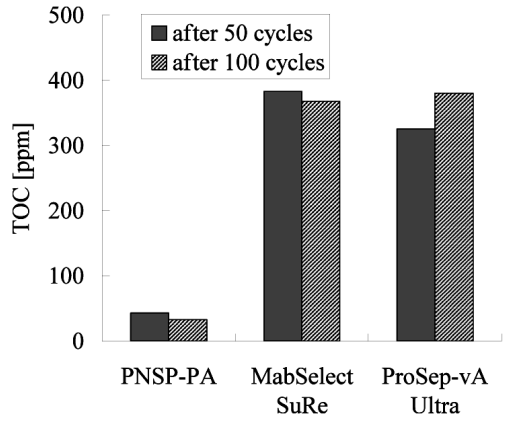

Figure 8. TOC values in PBS fractions.

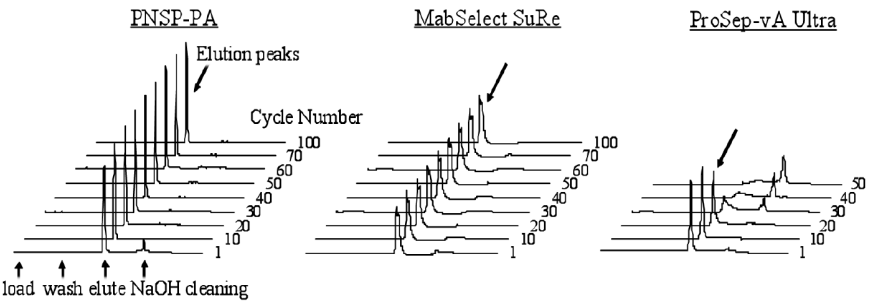

Figure 9. Influence of repeated 0.1 M sodium hydroxide cleaning.

Chemical stability in 100 cycles of operation in 0.1 M sodium hydroxide

Chemical stability was evaluated in 100 cycles of operation in 0.1 M sodium oxide, a solution commonly used for cleaning columns. Figure 9 shows the changes in elution peak shapes. The elution peak shape of PNSP-PA was the sharpest among the three. On the contrary, ProSep-vA Ultra began showing deterioration after 20 cycles and its peak was lost by 50 cycles. It was found that 20 mm of the column bed (corresponding to 20%) had been lost in the test. No such loss of the medium was observed for PNSP-PA.

Discussion

PNSP with a pore diameter of 100 nm showed an increase in the amount of PC groups according to an increase in molar ratio of PCS, as intended. The fact that total carbon contents did not change among these PNSP suggests that the amount of amino groups on PNSP must have decreased. The observed increases in the amounts of PC and amino groups according the order of PNSP (1:9, 100), PNSP (1:9, 70), and PNSP (1:9, 50) could be explained by differences in the surface areas of the corresponding silica, the starting material.

Comparing the amounts of non-specific adsorption on silica, NSP, and PNSP (9:1, 100), the adsorption level of PNSP (9:1, 100) was significantly lower than those of silica and NSP. Unmodified silica adsorbed IgG and lysozyme especially well. It was hypothesized that silica with negative charges attracted proteins with positive charges via ionic interaction. In contrast, NSP especially adsorbed albumin and IgG. This must have been because of positive charges derived from the amino groups of NSP. Therefore, the albumin and IgG electrically adsorbed on NSP and lysozyme with positive charges hardly repelled each other. This indicated that PNSP (9:1, 100) effectively inhibited the non-specific adsorption of proteins regardless of the charge of the proteins, which was ascribed to the PC groups.

As far as DBC was concerned, a larger value is preferred. DBC values increased as the amount of PC groups decreased. Since an excess amount of rprotein A was added to each PNSP reaction mixture, the amount of immobilized rprotein A seemed to be proportional to that of amino groups. Furthermore, the DBC value was supposed to be proportional to the amount of immobilized rprotein A. Therefore, the DBC results were understandable considering the balance of PC and amino groups.

PNSP (1:9, 70)-PA showed the largest DBC value among the three PNSP-PA. One reason why the 70-nm type had a larger DBC value was the differences in the levels of immobilized rprotein A. The contrary fact that the 70-nm type had a larger DBC value than the 50-nm type needs some explanation. One possible answer is steric hindrance between the pores and model proteins used, rprotein A (Mr : 55 kDa) and IgG (Mr: 150 kDa).

The suppression of non-specific adsorption was the main objective of the study. These results regarding non-specifically adsorbed HSA for PNSP-PA with different molar ratios of PC and amino groups suggest that even a small amount of PC groups can efficiently prevent the non-specific adsorption of HSA. As described earlier, a large DBC was preferred for protein A affinity chromatographic media. The results of comparisons performed in the study indicated the PNSP (1:9, 70) was the most optimized among the tested, as it showed the largest DBC value and still possessed a small amount of PC. The PNSP (1:9, 70) was therefore chosen as a candidate for further comparative studies.

From the DBC values observed in the three media, PNSP-PA, MabSelect SuRe, and ProSep-vA Ultra, the slow diffusion kinetics of the soft polysaccharide support, MabSelect SuRe, were somewhat expected. Although the amount of data was limited, it seems that silica has the best compatibility with a high flow rate.

From the result of chemical stability in 100 cycles of operation in 6 M guanidine-HCl, the yield of PNSP-PA was the highest of all the media, which did not contradict the DBC results. As for the TOC value of PNSP-PA, the structure of the PC groups seemed to contribute to the distinctive differences in the non-specifically adsorbed substances. In contrast, PCAF-PA had the disadvantage of its high back pressure, which was about two times higher than that of MabSelect SuRe. However, it was thought this was not a big problem because it did affect its performance. The amounts of rprotein A leakage in the eluted IgG fractions were not quantified because this would require some study before its application to the preparation of therapeutic antibodies, including the evaluation of leakage.

In general, siliceous materials, such as silica and glass, dissolved in basic solutions. It was interesting that PNSP-PA showed no change in peak shape during 100 cycles in 0.1 M sodium hydroxide, indicating that PNSP-PS was more stable in basic solutions than ProSep-vA Ultra, a glass-based medium.

Conclusion

PNSP-PA was developed by taking advantage of the characteristics of silica and PC groups and showed higher performance with regard to DBC and non-specific protein adsorption than other conventional media designed for protein purification. In addition, PNSP-PA showed adequate chemical stability under cleaning conditions with 0.1 M sodium hydroxide. PNSP-PA was found to be promising for fields requiring high-throughput and high-purity purification. Studies of its application and further improvement are necessary.

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