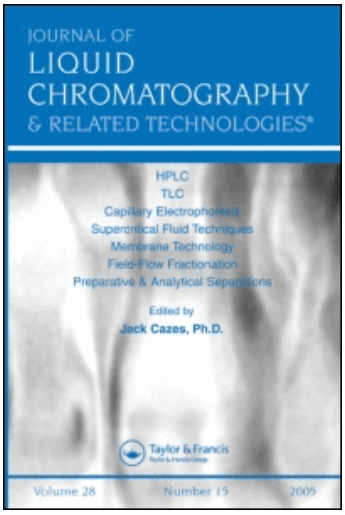

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

COMPARISON OF LYSOZYME ADSORPTION TO IMMOBILIZED CIBACRON BLUE 3GA USING VARIOUS MEMBRANE SUPPORTS

Shing-Yi Suen[a]; Rui-Long Chen[a]; Yi-Da Tsai[a]
[a] Department of Chemical Engineering, National Chung Hsing University, Taichung, Taiwan

Online publication date: 01 October 2000

To cite this Article Suen, Shing-Yi , Chen, Rui-Long and Tsai, Yi-Da(2000) 'COMPARISON OF LYSOZYME ADSORPTION TO IMMOBILIZED CIBACRON BLUE 3GA USING VARIOUS MEMBRANE SUPPORTS', Journal of Liquid Chromatography & Related Technologies, 23: 2, 223 — 239
To link to this Article: DOI: 10.1081/JLC-100101447
URL: http://dx.doi.org/10.1081/JLC-100101447

J. LIQ. CHROM. & REL. TECHNOL., 23(2), 223–239 (2000)

COMPARISON OF LYSOZYME ADSORPTION TO IMMOBILIZED CIBACRON BLUE 3GA USING VARIOUS MEMBRANE SUPPORTS

Shing-Yi Suen,* Rui-Long Chen, Yi-Da Tsai

Department of Chemical Engineering
National Chung Hsing University
Taichung, Taiwan 402

ABSTRACT

Lysozyme adsorption to immobilized Cibacron Blue 3GA using different membrane supports, regenerated cellulose membrane discs, and polysulfone hollow fibers, is investigated in this study. The relevant adsorption properties using traditional chromatographic beads were also measured for comparison. The experimental results demonstrated distinct adsorption properties for different solid supports. The membrane discs possessed the best thermal resistance among the three adsorbents assessed, which essentially results from their higher thermal stability. Moreover, the saturation capacity of lysozyme onto membrane discs was found close to the ligand density for gel beads, whereas the hollow fibers were of much greater saturation capacity.

INTRODUCTION

Affinity chromatography is a general purification technique used in the laboratories and industries. Up to now, many materials such as biomolecules, dye, and metal ions, have been immobilized onto solid supports as ligand for affinity separation. Among those materials, the so-called "group-specific affinity ligands" are frequently adopted due to their low price and capability of binding with broad species.

One of the most popular and commercially-available group-specific ligands is Cibacron Blue, a dye which can bind with NAD-cofactor enzymes, coagulation proteins, interferon, albumin, and even metal ions. Much attention has consequently been focused on the practical application of Cibacron Blue, especially its adsorption properties with proteins and metal ions. In addition, since affinity chromatography is usually carried out in columns packed with porous beads, the use of microbeads as adsorbents was emphasized in recent studies.[1-4]

In the past decade, the use of membrane materials as solid supports has demonstrated its potential in overcoming severe mass-transfer limitations for traditional column techniques.[5,6] Accordingly, more and more studies were attempted to improve the application of Cibacron Blue-immobilized affinity membranes.[7-12] However, very few of these researches compared the adsorption behaviors of the same molecule or ion using different types of membranes or microbeads as solid supports in Cibacron Blue affinity techniques. It was recently shown that biomolecules can have distinct adsorption properties on different solid supports such as membrane discs and gel beads.[13]

In order to determine a proper solid support, the basic adsorption properties for different membranes as well as chromatographic beads need to be extensively evaluated. Therefore, the objective of this study is to investigate the properties of lysozyme adsorption onto the immobilized Cibacron Blue 3GA under the same conditions, except for different solid supports. The solid supports compared in this work included membrane discs, hollow fibers, and gel beads, all commercially-available and ready-to-use.

EXPERIMENTAL

Materials

The solid supports employed in this work were Blue Sepharose CL-6B from Pharmacia Biotech AB (Uppsala, Sweden) as gel beads, regenerated cellulose filters from Sartorius AG (Goettingen, Germany) as membrane discs, and polysulfone microfiltration hollow fibers from A/G Technology (Needham, MA, USA). The basic properties for these supports provided from the manufacturing companies are listed in Table 1.

Cibacron Blue 3GA (C9534) and chicken egg white lysozyme (L6876, MW 14300) were purchased from Sigma Chemical (St. Louis, MO, USA). The loading buffer for adsorption was 50 mM Tris-HCl, pH 7, with 0.005% NaN_3. The elution buffer was 1 M KCl in Tris-HCl. Both buffers were filtered through 0.2 μm Nylon membranes (Lida Manufacturing, Kenosha, WI, USA). Lysozyme solution was made with loading buffer and filtered by 0.45 μm filters (Millex-HV, Millipore, Bedford, MA, USA).

Table 1

Basic Properties of Solid Supports Used in this Work

	Gel Beads	Membrane Discs	Hollow Fibers
Material	Cross-linked agarose	Regenerated cellulose	Polysulfone
Dimension	45-165 μm particle diam. (wet) (bead swelling in distilled water: 4-5 cm^3g^{-1})	25 mm diam. 80 μm thickness	0.75 mm inner diam. 1.3 mm outer diam. 15.5 cm length
Pore size	Not available	0.45 μm	0.65 μm
Porosity	0.00094 (dry)[a]	0.45 (0.36 ~ 0.67)	0.8
Temperature stability	4-40°C	Up to 180°C	Up to 50°C
pH stability	4-12	Not available	2-13

[a] Data measured by surface area and pore size analyzer.

Ligand Immobilization

The product of Blue Sepharose CL-6B has Cibacron Blue 3GA immobilized on the surface and is ready for use. On the other hand, the immobilization of Cibacron Blue 3GA onto membrane discs and hollow fibers needs to be conducted in this work.

Surface modification is also required before the binding of Cibacron Blue 3GA onto polysulfone hollow fibers.

The method of surface modification by Klein *et al*[14] using hydroxyethyl cellulose was employed with longer reaction time to fit the purpose of this work.

As to the immobilization of Cibacron Blue 3GA for both regenerated cellulose membrane discs and cellulose-modified hollow fibers, the method presented by Liu and Fried[8] and based on the binding of triazine group of Cibacron Blue 3GA with the OH groups of cellulose was adopted.

Batch Equilibrium Experiments

For batch adsorption equilibrium experiments, constant volume of lysozyme solution of a given concentration and constant weight of adsorbents were placed in a container: 5 mL solution and 0.1 g Blue Sepharose CL-6B for gel beads; 10 mL solution and 5 dry discs with immobilized Cibacron Blue 3GA for membrane discs; 25 mL solution and one dry hollow fiber with immobilized Cibacron Blue 3GA for hollow fiber experiment. The protein solution with solid adsorbents was incubated at a fixed temperature for 12 h to reach equilibrium. Centrifugation was conducted for the sample of gel bead suspension before the measurement of protein solution concentration. Lysozyme concentration in the solution was determined using a UV-Vis spectrophotometer (Model U-2000, Hitachi, Tokyo, Japan; UV-1601, Shimadzu, Auburn, Australia) under the wavelength of 280 nm. The extinction coefficient of lysozyme, $E^{280nm}_{1\ mg/ml}$, is 2.65.[1,2]

The amount of protein adsorbed was determined from the difference between the protein concentration initially added and that left in the supernatant afterwards. After each batch experiment, the membrane discs and hollow fibers were washed thoroughly with elution buffer to remove the bound protein out of adsorbents. These membrane adsorbents were restored in the loading buffer for repeated use.

The experiments were performed at 4, 25, and 45°C, respectively. The experiments for gel beads and membrane discs at 25°C have been conducted and published earlier.[13] Those data are directly used in this work.

RESULTS AND DISCUSSION

Adsorption Isotherms

The isotherms of lysozyme adsorption to immobilized Cibacron Blue 3GA using different types of membrane supports were measured in this work. In addition, the corresponding isotherms using gel beads were also investigated for comparison. Only the temperature effect was discussed in this study. Other effects from the variation of buffer composition, pH and ionic strength have been reported in the literature for gel beads.[1,3]

In the work of Boyer and Hsu,[1] the adsorption of lysozyme onto Blue Sepharose CL-6B was found to be insensitive to the buffer system and pH variation (from 7 to 8.5), whereas increasing the ionic strength resulted in decreased protein binding. It should be noted that the binding of lysozyme with Cibacron Blue is due to both ionic and hydrophobic interactions. Because lysozyme has an isoelectric point of 11, lysozyme molecule would be cationic at pH values far

below 11 (for example, under pH 9) and the change of pH in that range would notfurther influence its ionic binding. In addition, in the pH range of 5.5 to 8, the pH dependence of protein binding is mainly due to the titration of histidine residues. This interpretation could explain the pH independence of lysozyme binding due to its low histidine content.[1] On the other hand, the addition of ionic strength in the buffer would lead to an adsorption competition between other ions and lysozyme molecule since the binding of lysozyme to Cibacron Blue is partly due to ionic interaction. Therefore, low ionic strength should be used in the adsorption process in order to enhance the lysozyme adsorption.

According to the above discussion, the optimal buffer condition for lysozyme adsorption should be a buffer with low salt concentration and with pH value under 9. The loading buffer hired in this work followed this optimal condition with pH 7. The purpose of not using lower pH values is to prevent the pH dependence of lysozyme adsorption and also to avoid the difficulty of lysozyme desorption from the solid supports. In this work, the loading buffer with higher salt concentration was simply used as the desorption agent.

Two lower temperatures of 4°C and 25°C were used in this work, both being the most frequently adopted temperatures in the bioseparation experiments. Another higher temperature of 45°C was also employed. The main reason for choosing 45°C is that its increment to 25°C is almost identical to that of 25°C to 4°C. It should be noted that the thermal stabilities for gel beads and hollow fibers are limited up to 40°C and 50°C, respectively, according to the report of the manufacturing company (shown in Table 1). 45°C is accordingly very close or even beyond the thermal stability limit and should be avoided. However, the limit of thermal stability reported by the manufacturer is only for solid materials. The surface modification of solid materials for ligand immobilization in this work may have altered the surface properties of solid supports. Therefore, the use of a temperature close to the reported thermal stability limit may provide the information to judge if the thermal stability limit is significantly altered by the immobilization procedure.

The experimental results are illustrated in Figures 1 to 3 for gel beads, membrane discs, and hollow fibers, respectively. In this study, bound protein amount was based on solid adsorbent volume. To distinguish the effects of specific and nonspecific binding, blank membrane discs, and the hollow fibers modified solely with hydroxyethyl cellulose in the absence of Cibacron Blue 3GA were used for lysozyme adsorption at room temperature. The nonspecific adsorption capacities at high equilibrium concentration were found to be 240 μM for membrane discs and 130 μM for hollow fibers. These values are relatively small as compared to the results in Figures 2 and 3 (the specific adsorption). Therefore, in the following analyses, the adsorption capacity from nonspecific binding was neglected.

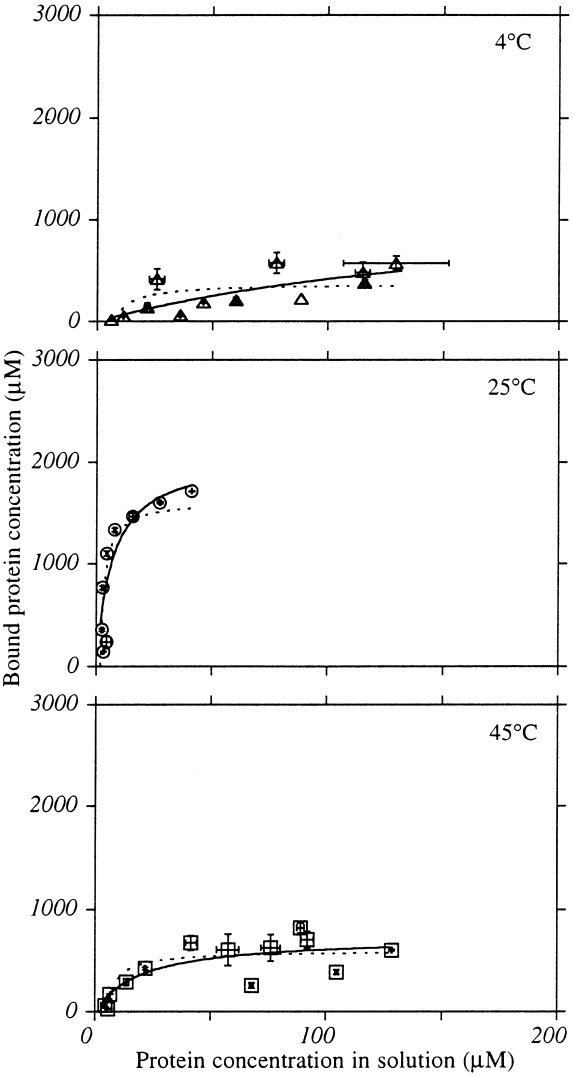

Figure 1. Adsorption isotherms for the batch equilibrium binding of lysozyme to Blue Sepharose CL-6B. Error bars span 2 standard deviations. Solid line: Langmuir model; dashed line: Suen model.

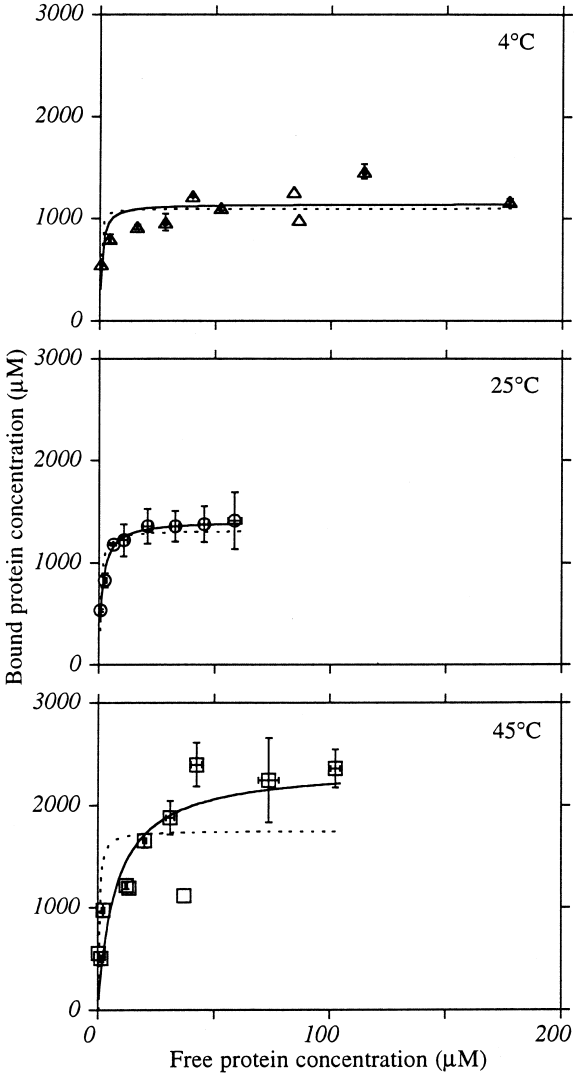

Figure 2. Adsorption isotherms for the batch equilibrium binding of lysozyme to Cibacron Blue 3GA immobilized onto regenerated cellulose membrane discs. Error bars span 2 standard deviations. Solid line: Langmuir model; dashed line: Suen model.

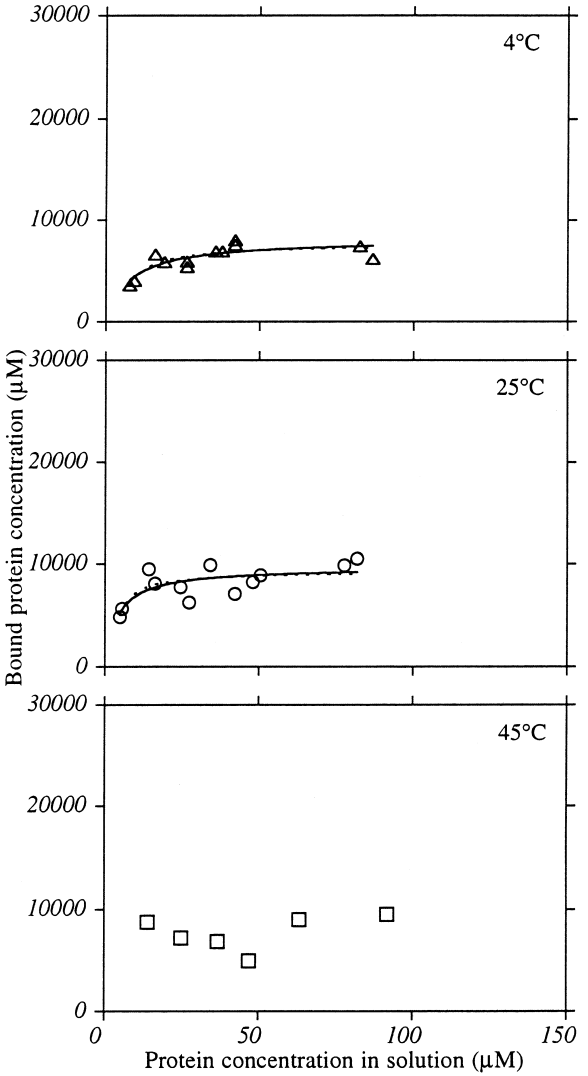

Figure 3. Adsorption isotherms for the batch equilibrium binding of lysozyme to Cibacron Blue 3GA immobilized onto modified polysulfone hollow fibers. Error bars span 2 standard deviations. Solid line: Langmuir model; dashed line: Suen model.

Two single-solute isotherm models were used to fit the experimental data:

$$\text{the Langmuir model} \quad c_s = \frac{c\,c_1}{K_d + c}, \tag{1}$$

$$\text{and the Suen model}^{15} \quad c_s = \begin{cases} 0 & c \le \dfrac{\hat{K}_d}{c_1} \\ c_1 - \dfrac{\hat{K}_d}{c} & c > \dfrac{\hat{K}_d}{c_1} \end{cases} \tag{2}$$

where c is the protein concentration in solution (μM), c_s is the adsorbed protein concentration based on solid adsorbent volume (μM), c_1 is the saturation capacity of protein based on solid volume (μM), K_d is the dissociation equilibrium constant for the Langmuir model (μM), and $\hat{K}_d$ is the dissociation equilibrium constant for the Suen model [$(\mu M)^2$]. The fitted curves are plotted with solid lines for the Langmuir model and dashed lines for the Suen model. The fitted parameter values are listed in Table 2. It should be noted that the 45°C data of hollow fibers, more closely resembling a straight line of zero slope, could not be reasonably fitted by both models and consequently no fitting results are shown in the figure and table. By comparing the fitting results, the Langmuir model showed a slightly better fitting for gel beads and membrane discs, but the Suen model displayed the same degree of agreement in the results for hollow fibers.

The isotherm results show that hollow fibers exhibited the highest adsorption capacities among all the three supports. This is very possibly due to the fact that the hollow fiber design usually provides higher specific surface area and results in higher related adsorption capacity. The saturation capacities of lysozyme onto membrane discs were found close to those for gel beads, except for the 45°C case. The highest adsorption capacity occurred at 25°C for the gel beads and so did the hollow fibers. However, the results using membrane discs displayed a different trend, in which the capacity of lysozyme adsorption increased with temperature.

The reason for this difference may be that the temperature of 45°C is very close to or even beyond the thermal stability limit of gel beads and hollow fibers and consequently causes damages to the solid materials. The surface modifications during ligand immobilization are proved to have insignificant effects in changing the thermal duration of solid supports. The regenerated cellulose membranes, on the other hand, offer better thermal resistance up to 180ºC. Accordingly, higher temperature may help more protein adsorbed onto the membrane discs without thermal stability problems.

Table 2

The Fitted Parameter Values for Different Solid Supports

		Langmuir Model	Suen Model
Gel beads	4°C	$c_l = 1200 \pm 1000$ μM $K_d = 200 \pm 230$ μM R = 0.75	$c_l = 370 \pm 50$ μM $K_d = 2600 \pm 800$ $(\mu M)^2$ R = 0.61
	25°C	$c_l = 2100 \pm 200$ μM $K_d = 6.8 \pm 1.8$ μM R = 0.84	$c_l = 1600 \pm 100$ μM $K_d = 2900 \pm 400$ $(\mu M)^2$ R = 0.82
	45°C	$c_l = 730 \pm 110$ μM $K_d = 20 \pm 11$ μM R = 0.78	$c_l = 600 \pm 40$ μM $K_d = 2700 \pm 500$ $(\mu M)^2$ R = 0.77
Membrane discs	4°C	$c_l = 1100 \pm 50$ μM $K_d = 0.73 \pm 0.35$ μM R = 0.73	$c_l = 1100 \pm 50$ μM $K_d = 190 \pm 60$ $(\mu M)^2$ R = 0.64
	25°C	$c_l = 1400 \pm 70$ μM $K_d = 1.4 \pm 0.4$ μM R = 0.85	$c_l = 1300 \pm 60$ μM $K_d = 570 \pm 120$ $(\mu M)^2$ R = 0.79
	45°C	$c_l = 2400 \pm 200$ μM $K_d = 7.8 \pm 3.4$ μM R = 0.84	$c_l = 1800 \pm 100$ μM $K_d = 680 \pm 210$ $(\mu M)^2$ R = 0.61
Hollow fibers	4°C	$c_l = 8100 \pm 600$ μM $K_d = 7.6 \pm 2.6$ μM R = 0.83	$c_l = 7600 \pm 300$ μM $K_d = 31000 \pm 6000$ $(\mu M)^2$ R = 0.86
	25°C	$c_l = 9700 \pm 700$ μM $K_d = 4.0 \pm 1.8$ μM R = 0.73	$c_l = 9300 \pm 500$ μM $K_d = 21000 \pm 6000$ $(\mu M)^2$ R = 0.74
	45°C	-----	-----

R = Correlation coefficient.

As to the analyses of the binding strength between protein and ligand for different supports, the data of dissociation equilibrium constant in Table 2 were compared. Among these three supports, the lowest values of dissociation equilibrium constants were shown on the membrane discs, irrelevant of the employed isotherm model. The reciprocal of the dissociation constant indicates a measure of the association strength. That is, the binding of lysozyme with immobilized Cibacron Blue 3GA was strengthened when membrane discs were used. On the other hand, different isotherm models displayed different magnitudes of dissociation equilibrium constants for the results of gel beads and hollow fibers. It leads to difficulties in comparing the affinity strength for these two supports.

Scatchard Plots

To analyze the adsorption homogeneity, the Scatchard plots of experimental data are displayed in Figures 4 to 6. The Scatchard curves for both isotherm models used in this work correspond to the following equations:

$$\text{the Langmuir model} \quad \frac{c_s}{c} = \frac{c_l}{K_d} - \frac{1}{K_d} c_s , \tag{3}$$

$$\text{and the Suen model}^{15} \quad \frac{c_s}{c} = \frac{c_l}{\hat{K}_d} c_s - \frac{1}{\hat{K}_d} c_s^{\,2} . \tag{4}$$

The formation of the Langmuir model is based on the ideal assumption of homogeneous adsorption, *i.e.* one-to-one binding in a single adsorption layer. Therefore, the negative-slope linearity shown in Eq. (3) for the Langmuir model becomes an index for adsorption homogeneity. The Scatchard curves which do not demonstrate negative-slope straight lines will represent the heterogeneous adsorption. For example, the Scatchard plot of the Suen model in Eq. (4), illustrating a concave-down curve, is an example of heterogeneous adsorption. A concave-down curvature in the Scatchard curve usually reflects the positive cooperativity between the interacting sites. The Scatchard plots of the model results using the parameter values listed in Table 2 are also presented in Figures 4 to 6 to illustrate the adsorption homogeneity of experimental data.

In Figure 4, most Scatchard data of gel beads were scattered, except that the 25°C data resembled a concave-down curve. Furthermore, by comparison of experimental data with model results, the Suen model showed a better agreement than the Langmuir model. This indicates that the lysozyme adsorption onto dye-immobilized gel beads is heterogeneous. In addition, the scattered nature of the experimental data may imply the changes of adsorption mechanisms during the binding process.

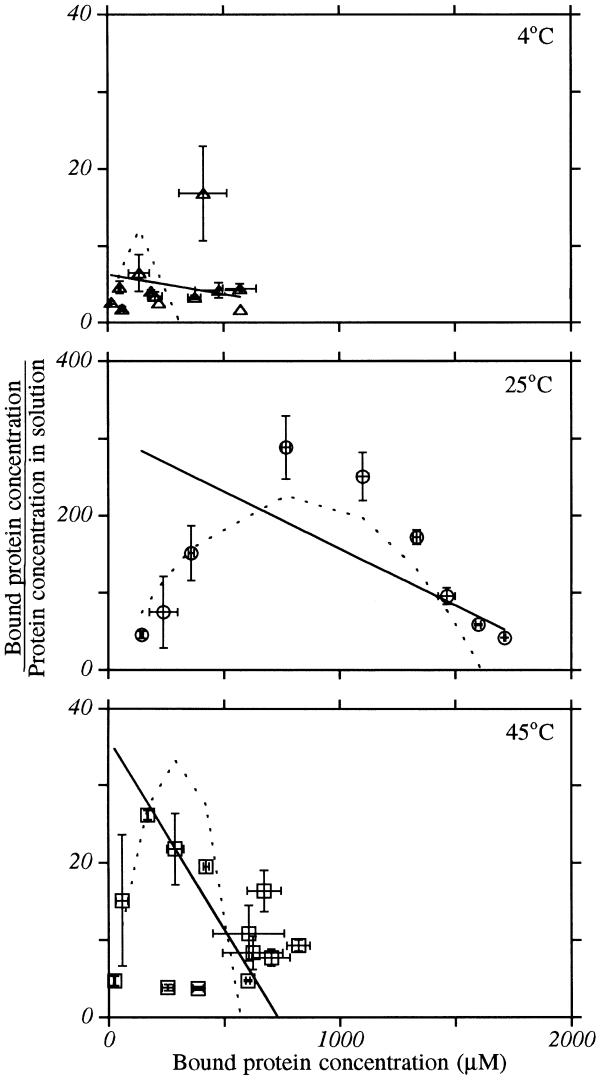

Figure 4. Scatchard plots for adsorption isotherms of lysozyme to Blue Sepharose CL-6B. Error bars span 2 standard deviations. Solid line: Langmuir model; dashed line: Suen model.

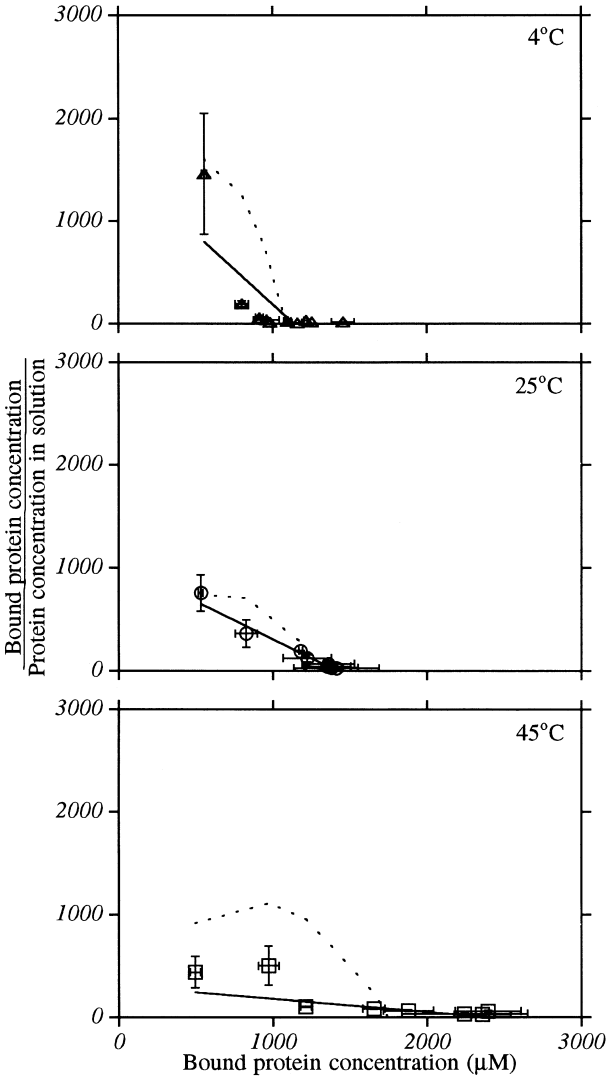

Figure 5. Scatchard plots for adsorption isotherms of lysozyme to Cibacron Blue 3GA immobilized onto regenerated cellulose membrane discs. Error bars span 2 standard deviations. Solid line: Langmuir model; dashed line: Suen model.

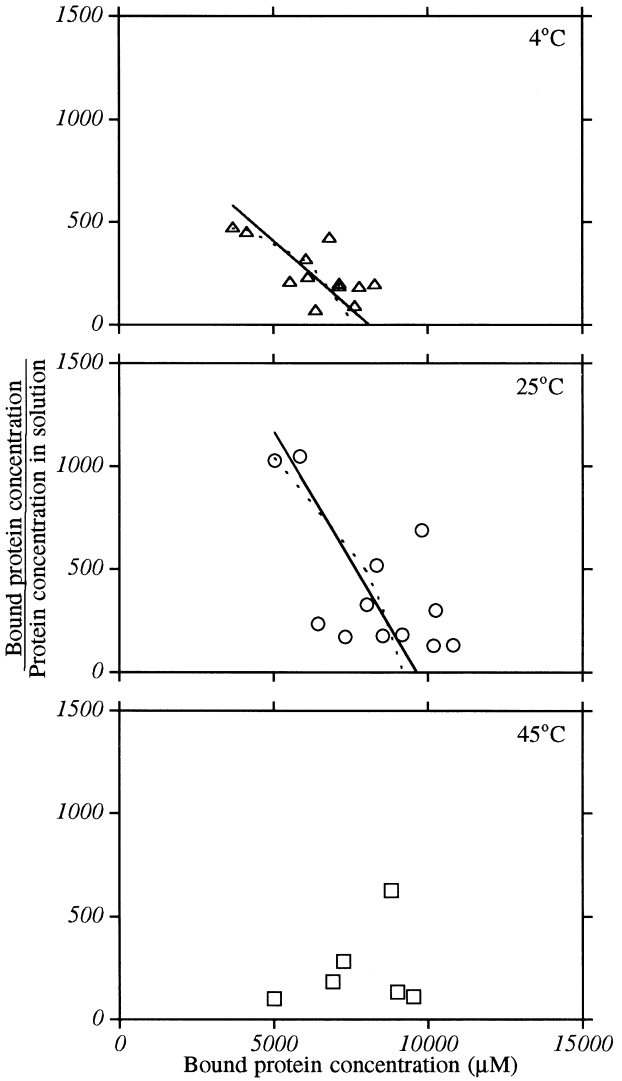

Figure 6. Scatchard plots for adsorption isotherms of lysozyme to Cibacron Blue 3GA immobilized onto modified polysulfone hollow fibers. Error bars span 2 standard deviations. Solid line: Langmuir model; dashed line: Suen model.

In contrast to the results of gel beads, the Scatchard plots for membrane discs in Figure 5 more closely resembled negative-slope lines and the Langmuir model is obviously a better choice for the data fitting as shown in Figure 5. As to the Scatchard plots of hollow fibers, the experimental data were all scattered and did not resemble any of the two model results. Therefore, similar to the results of gel beads, the lysozyme adsorption onto dye-immobilized hollow fibers is heterogeneous and the more complicated adsorption mechanisms than simple concave-down curves may have dominated the binding process. In summary, different adsorption heterogeneity has occurred for lysozyme adsorption onto different solid supports. The adsorption onto gel beads and hollow fibers can be categorized to heterogeneous adsorption, whereas adsorption onto membrane discs is more likely homogeneous.

Ligand Density and Utilization Percentage

To further explore the binding behaviors, the ligand density and protein adsorption capacity for different supports are compared. For chromatographic beads, Blue Sepharose CL-6B, the ligand density reported by the manufacturer is 1700 to 2300 μM based on the drained gel. This data can be directly compared to the saturation capacities obtained in this work, which are 370 to 1200 μM for 4°C; 1600 to 2100 μM for 25°C; 600 to 730 μM for 45°C. Only the result at 25°C is close to the ligand density; that is, the lysozyme-dye binding may be considered a single-site interaction at room temperature and almost 100% of ligand sites were utilized. Decreasing or increasing the temperature may retard the protein adsorption such that a certain amount of ligand sites was not used. The overall utilization range is about 20% to 90%.

The ligand density for membrane discs was reported to be about 46000 μM in the author's previous work,[13] based on solid volume. This value is a lot greater than the saturation capacity of 1100 to 2400 μM for membrane discs as listed in Table 2. The utilization percentage is very low, only 2-5%. In other words, a large portion of ligand sites was not utilized and still empty during the process of adsorption. This phenomenon may be because the ligand sites were too crowded for the relatively big protein molecule to attach. Furthermore, spacer arm was not used in this study during the process of ligand immobilization onto the membrane discs, which may also make the protein molecule difficult to touch the solid membrane surface due to the resistance of liquid-film mass transfer.

As to the measurement of ligand density for hollow fibers, the method used in the literature[2] can not be applied because the polysulfone hollow fibers used in this study were not completely dissolved at high concentration of HCl and high temperature. The value of ligand density for hollow fibers is accordingly not obtainable, which makes the comparison with the saturation capacities unfeasible.

CONCLUSIONS

The adsorption of lysozyme to immobilized Cibacron Blue 3GA using different membrane supports: membrane discs, hollow fibers, and the traditional gel beads, is investigated in this study. The experimental results show that the adsorption properties were not identical for different solid supports. First, it is found that the regenerated cellulose membrane discs offered the best thermal resistance compared to the other two adsorbents, and the increase of temperature would increase the saturation capacity of lysozyme adsorbed onto the membrane discs. Therefore, membrane discs are suggested to be a better choice for the dye-affinity separation processes conducted above room temperature.

Moreover, membrane adsorbents, especially membrane discs, are usually considered to be with much lower ligand density than conventional chromatographic particles, which significantly deteriorates their potential for practical use even though they have demonstrated better mass transfer characteristics. However, the results in this work show that the saturation capacities of lysozyme onto membrane discs were close to the ligand density of commercial gel beads, and the saturation capacities for hollow fibers were even much greater. Consequently, the capacities for adsorptive membranes may be competitive to the capacities for gel beads used in traditional chromatography. According to the results in this work, hollow fibers should be the best choice for the adsorption process of large capacity.

Finally, the ligand utilization ratio for membrane discs is found to be very low, compared to the commercial gel beads. To raise its ligand utilization, the use of spacer arm may be suggested.

ACKNOWLEDGMENT

This research was sponsored by the National Science Council of the Republic of China (NSC 85-2214-E-005-009 and NSC 86-2214-E-005-004).

REFERENCES

1. P. M. Boyer, J. T. Hsu, Biotechnol. Tech., **4**, 61-66 (1990).

2. P. M. Boyer, J. T. Hsu, Chem. Eng. Sci., **47**, 241-251 (1992).

3. G. M. S. Finette, Q.-M. Mao, M. T. W. Hearn, J. Chromatogr. A, **763**, 71-90 (1997).

4. M. Kocakulak, A. Denizli, A. Y. Rad, E. Piskin, J. Chromatogr. B, **693**, 271-276 (1997).

5. S. Brandt, R. A. Goffe, S. B. Kessler, J. L. O'Connor, S. E. Zale, Bio/Technology, **6**, 779-782 (1988).

6. K.-G. Briefs, M.-R. Kula, Chem. Eng. Sci., **47**, 141-149 (1992).

7. W. Guo, Z. Shang, Y. Yu, L. Zhou, J. Chromatogr. A, **685**, 344-348 (1994).

8. H.-C. Liu, J. R. Fried, AIChE, **40**, 40-49 (1994).

9. M. Weissenborn, B. Hutter, M. Singh, T. C. Beeskow, F. B. Anspach, Biotechnol. Appl. Biochem., **25**, 159-168 (1997).

10. A. Denizli, B. Salih, M. Y. Arica, K. Kesenci, V. Hasirci, E. Piskin, J. Chromatogr. A, **758**, 217-226 (1997).

11. M. Y. Arica, A. Denizli, B. Salih, E. Piskin, V. Hasirci, J. Membr. Sci., **129**, 65-76 (1997).

12. A. Denizli, D. Tanyolac, B. Salih, E. Aydinlar, A. Ozdural, E. Piskin, J. Membr. Sci., **137**, 1-8 (1997).

13. S.-Y. Suen, Y.-S. Chang, J. Chin. Inst. Chem. Engrs., **29**, 307-317 (1998).

14. E. Klein, E. Eichholz, D. H. Yeager, J. Membr. Sci., **90**, 69-80 (1994).

15. S.-Y. Suen, J. Chem. Technol. Biotechnol., **70**, 278-286 (1997).

Received January 15, 1999
Accepted May 18, 1999
Manuscript 4967