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Macroligand D-Alanyl-D-alanine-dextran for Vancomycin Purification

Equilibrium Binding Study

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ABSTRACT

Water soluble macroligands for vancomycin purification in affinity ultrafiltration have been prepared by coupling the ligand D-alanyl-D-alanine to dextran activated by tosyl chloride, carbonyldiimidazole, and chloroformate, respectively. Centrifugal ultrafiltration has been used to study the equilibrium binding of vancomycin for the macroligands. The affinity binding can be described as Langmuir type adsorption and is strongly affected by temperature. The binding between vancomycin and macroligand is an unusual endothermic process that binding capacity of macroligand increases with temperature. Vancomycin has also been successfully purified from fermentation liquor using the macroligand in a centrifugal ultrafiltration device.

Index Entries: Immobilized D-alanyl-D-alanine; vancomycin purification; affinity ultrafiltration; equilibrium binding.

INTRODUCTION

Glycopeptide antibiotic vancomycin was the first to be discovered in the vancomycin group antibiotics in 1956 (*1*). Over ten members of this group have since been reported, all of which possess closely related chemical structures and are active against gram-positive bacteria. It is now a clinically important therapeutic agent used for the treatment of infection

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owing to methicillin resistant *Staphylococcus sp.* (2). The primary mode of antimicrobial action of vancomycin is inhibition of cell wall synthesis. Vancomycin binds very tightly to peptides that contain D-alanyl-D-alanine at the free carboxyl end and prevents the polymerization of peptidoglycan, which is vital for the existence of gram-positive bacteria, during cell wall synthesis (3). Based on this affinity interaction, the purification of vancomycin and several other glycopeptide antibiotics have been carried out by affinity chromatography (4,5) and affinity aqueous two-phase extraction (6) using D-alanyl-D-alanine as an affinity ligand.

Recently, affinity membrane filtration has been demonstrated to purify proteins in batch, semibatch, and continuous operation successfully (7–10). This technique combines the high selectivity of affinity chromatography with the high volume processing capacity of membrane filtration, and therefore can be easily adapted to large-scale operation. A feature of affinity membrane filtration is that it can employ a macroligand to specifically adsorb the desired product so that it can be retained by the ultrafiltration or microfiltration membrane. The so-called macroligand consists of ligands covalently attached to a water soluble high molecular weight polymer or to a small size particle. Thus, the sizes of the desired product and macroligand together with the pore size of the filtration membrane will determine the efficiency and quality of the purification. Because of the low selectivity of membrane separation process, the larger size difference between the desired product and macroligand is more favorable for separation. For a solid macroligand, the steric hindrance and internal mass transfer resistance may limit the separation efficiency. By employing a water soluble macroligand, homogeneous binding can be achieved that reduces the steric hindrance and eliminates the mass transfer resistance. Therefore, the small size biomolecules such as vancomycin (mol wt ca 1500), which has a very specific affinity ligand is very suitable to be purified by affinity ultrafiltration using water soluble macroligands.

A thorough understanding of the binding of vancomycin for macroligand would clearly aid in the development of a properly designed affinity ultrafiltration system. In this work we attempted to prepare macroligands by coupling D-alanyl-D-alanine to dextran (mol wt ca 500,000) through various coupling agents and studied the binding properties, i.e., the affinity constants and maximal binding capacity of the macroligands for vancomycins. Further, we investigated the purification of vancomycin from fermentation liquor using the prepared macroligand.

MATERIALS AND METHODS

Materials

Vancomycin, D-alanyl-alanine, and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Dextran

(MW 500,000) and *p*-toluenesulfonic chloride (tosyl chloride) were obtained from Fluka Chemical Co. (Buchs, Switzerland). Carbonyldiimidazole and *p*-nitrophenyl chloroformate were obtained from Aldrich (Milwaukee WI) and Merck Chemical Co. (Darmstadt, Germany), respectively. All other chemicals were of analytical grade.

Macroligands Preparation

Dextran is a water soluble natural polymer of D-glucose and has plenty of hydroxyl groups that can be activated for ligand coupling. Coupling agents tosyl chloride, *p*-nitrophenyl chloroformate, and carbonyldiimidazole were employed to activate dextran, respectively. In order to achieve good activation yield, the activation process should be carried out in the absence of water. Dextran powder was dried at 100°C for 1 h and soaked in dry acetone. The dextran recovered from acetone was dried again at 100°C for 1 h. The activation was carried out by mixing 1.5 g dry dextran with 0.61 mmole coupling agents and 45 mL dry acetone. In the case of carbonyldiimidazole, activation was performed under nitrogen atmosphere for 1 h. For activation using tosyl chloride and *p*-nitrophenyl chloroformate, 0.1 mL pyridine was added into the activation solution as an organic base and the activation reactions lasted for 1 h and 24 h, respectively. The activated dextran was collected and washed thoroughly with dry acetone.

The second step in the process of macroligand preparation is ligand coupling to the activated dextran. One gram of activated dextran and 70 mg ligand D-alanyl-D-alanine were dissolved in 100 mL buffer solution and reacted at room temperature for 20 h. The buffer solutions employed for coupling ligand to tosyl chloride, *p*-nitrophenyl chloroformate, and carbonyldiimidazole activated dextrans were borate buffer (0.1*M*, pH 9.0), phosphate buffer (0.05*M*, pH 8.5), and carbonate buffer (0.15*M*, pH 10.0), respectively. At the end of ligand coupling reaction, 10 mL enthanolamine was added into the reaction solution to block the unreacted activation sites. The reaction solution was then filtered with 0.45 μm nylon membrane to remove the suspended impurities. The macroligand, D-alanyl-D-alanine-dextran, was recovered by diafiltration using a UF membrane of MWCO 20,000 and 500 mL phosphate buffer (0.05*M*, pH 7.0) and concentrated to 10 mL. The concentrated macroligand solution was freezed at −20°C until use.

Affinity Binding

The binding of vancomycin to the prepared macroligand was studied using a centrifugal UF device Centricon 30 (Amicon Co., Danvers, MA). Molecular weight cutoff of the membrane in Centricon 30 was 30,000. Vancomycin (mol wt ca 1500) can easily pass through the membrane during ultrafiltration by centrifugation, whereas the macroligand will be totally retained. The concentrated macroligand solution of 0.1 mL and 1.9

mL phosphate buffer (0.05*M*, pH 7.0) containing various amounts of vancomycin were added into Centricon 30. Centricon was then incubated in the rotor for 1 h and centrifuged at 4000 rpm for 15 min at a constant temperature using Beckman centrifuge R2-21. Vancomycin concentration in the filtrate was considered as equilibrium concentration. The difference between the initial and equilibrium concentration of vancomycin was used to calculate the amount of vancomycin bound to macroligand.

Purification of Vancomycin from Fermentation Liquor

Vancomycin can be produced by *Streptomyces orientalies* (ATCC 19795) (*1*). When this microorganism was cultivated in this study, however, no vancomycin was detected in the liquor. Pure vancomycin was then added into the fermented liquor that contained biomass, molasses, and inorganic salts. The simulated liquor containing 2 mg/mL vancomycin was first filtered with 0.45-μm membrane to remove the cell mass. The clear filtrate was then ultrafiltered with UF membrane of 20,000 mol-wt cutoff to remove the dissolved macromolecules. The purification steps, i.e., adsorption, washing, and elution were carried out in Centricon 30 at 25°C. The liquor filtrate of 2 mL was mixed with about 5 mg macroligand in Centricon 30. After adsorption, the macroligand was washed twice with phosphate buffer and the vancomycin was eluted with 0.5*M* ammonium solution. Vancomycin concentration in the filtrates of each purification steps were analyzed with HPLC.

Assay Technique

The concentration of ligand D-alanyl-D-alanine was determined by TNBS method (*11*). Vancomycin concentration in buffer solution was determined by UV adsorbance at 280 nm using Shimadzu spectrophotometer (model UV-160A).

Vancomycin concentration in the fermentation liquor was analyzed using Gilson HPLC system. Reverse-phase C-18 column of 25 cm was used. The mobile phase was composed of 75% 0.05*M* pH 7.0 phosphate buffer and 25% acetonitrile.

RESULTS AND DISCUSSION

Macroligands Preparation

The ligand density of macroligands prepared from the activated dextrans is shown in Table 1. The chloroformate activated dextran gives the highest ligand density, about 162 mmole D-alanyl-D-alanine was coupled to 1 g of dry dextran. This value corresponds to about 80 D-alanyl-D-alanine molecules were coupled to one dextran molecule. In other words,

Table 1
Ligand Density and Maximal Binding Capacity of Macroligands

Macroligands activated by	Ligand density, μ mole/g dextran	Maximal binding capacity, Q_{max}, μ mole/g dextran					Binding ratio
		4°C	10°C	20°C	30°C	$Q_{max\text{-}average}$	
Tosylchloride	102	120	120	128	125	123	1.2
Carbonyldiimidazole	132	183	187	180	182	183	1.4
Chloroformate	162	234	217	230	224	226	1.4

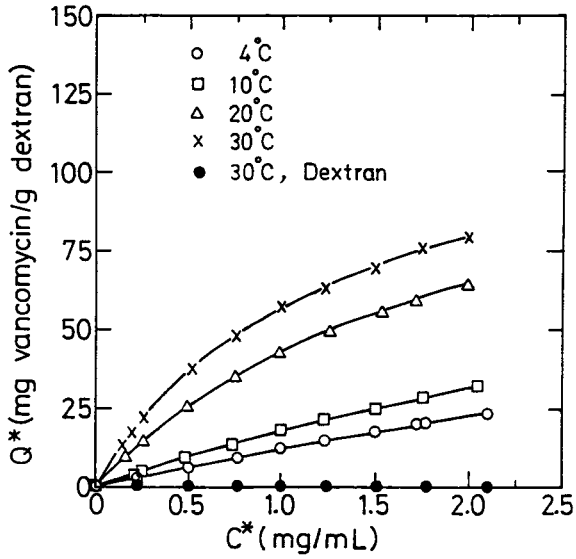

Fig. 1. Binding isotherms of vancomycin for the macroligand prepared from tosyl chloride activated dextran; Q^*, the amount of vancomycin bound to macroligand; C^*, the equilibrium vancomycin concentration.

only 3% of the glucose units in one dextran molecule were coupled with ligand. The low ligand density is mainly attributed to the activation of dextran in dry organic solvent. Since dextran is insoluble in organic solvent, only the surface hydroxyl groups of dextran powder are available for activation.

Affinity Binding

The vancomycin binding to the water soluble macroligands in phosphate buffer were studied in a centrifugal UF device. In order to explore the nonspecific binding and vancomycin retention by concentration polarization layer formed on UF membrane surface, a control run using unmodified dextran was carried out. Figure 1 shows the affinity binding isotherm of dextran and macroligand prepared by using tosylchloride as an activation agent. The other two macroligands prepared by using carbonyldiimidazole and chloroformate behave similarly. Dextran itself does

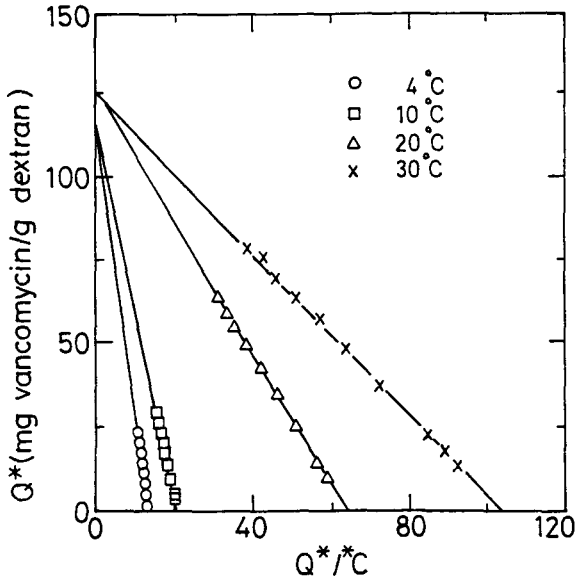

Fig. 2. Scatchard plot of Fig. 1 for the determination of affinity properties of macroligand.

not bind with vancomycin and the polarization layer has no appreciable effect on retarding the transport of vancomycin through the membrane. The amount of vancomycin binding to the macroligand increases with free vancomycin concentration and temperature. The binding isotherms of Fig. 1 can be replotted according to the method of Scatchard

$$Q^* = Q_{max} - K_d \cdot Q^* / C^* \quad (1)$$

where Q^* is the amount of vancomycin binding to macroligand and C^* is the free vancomycin concentration. Figure 2 shows typical results of such a plot. The linear plots indicate that vancomycin binding to the macroligands can be described by the Langmuir type adsorption. The maximal amount of vancomycin binding to the macroligands, Q_{max}, and the dissociation constant between vancomycin and macroligands, K_d can be obtained from the intercept and slope of such a plot, respectively. The binding properties of the three macroligands are listed in Tables 1 and 2.

As shown in Table 1, the Q_{max} is independent of temperature but dependent on the macroligands of different preparation since the Q_{max} represents the maximal binding capacity of macroligand. Assuming a 1:1 ratio of vancomycin to ligand, the maximal binding capacity would be expected to match the ligand density. This property could be examined by comparing the Q_{max} with ligand density of macroligands. In each case as shown in Table 1, the ligand binding capacity exceeded the amount of bound ligand by factors of 1.2–1.4. This property increases with the amount of bound ligands on the macroligand. Excess binding capacity was also observed by other workers (*5,12*) and attributed to the tendency of vancomycin to aggregate.

Table 2
Effect of Temperature on Association Constant, K_a

Temperature, °C	Macroligands activated by, K_a, $Lmole^{-1}$		
	Tosylchloride	Carbonyldiimidazole	Chloroformate
4	1.69×10^2	1.85×10^2	1.75×10^2
10	2.63×10^2	2.94×10^2	2.70×10^2
20	7.69×10^2	7.14×10^2	6.25×10^2
30	1.25×10^3	1.43×10^3	2.00×10^3

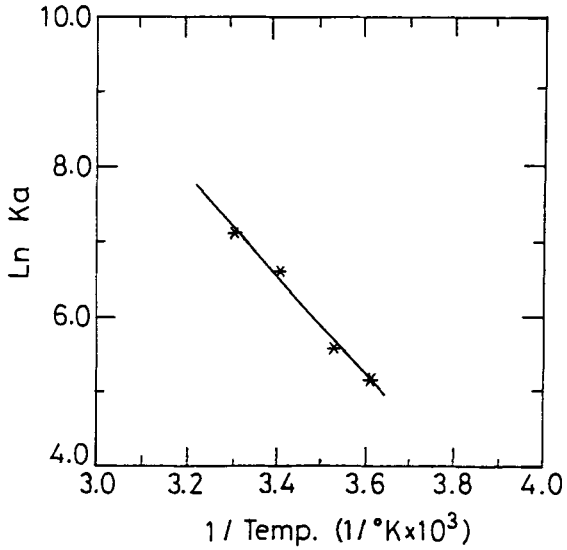

Fig. 3. The temperature dependency of the association constant of the macroligand prepared from tosyl chloride activated dextran.

The dissociation constant of vancomycin for macroligands calculated from Scatchard plot can be converted to association constant K_a and reported in Table 2. The affinity of the vancomycin for all three macroligands is of the same order of magnitude. This association constant is two orders of magnitude smaller than that of vancomycin for *N*-acetyl-D-alanyl-D-alanine (*13*) and for D-ala-D-ala-agarose (*4*), in which 6-carbon *N*-acyl chains between dipeptide and agarose was provided by the spacer arm. This should not be completely unexpected, since the spacer arm provided by the coupling agents used in this study is too short and the dextran structure itself may interfere the affinity binding of vancomycin to the ligand.

The temperature has a strong effect on the vancomycin binding for the macroligand, as shown in Fig. 1, where the binding increases with temperature. Based on van't Hoff equation, a plot of log K_a against 1/T (°K) is shown in Fig. 3. The enthalphy change for the affinity binding to

Table 3
Purification of Vancomycin Using Macroligand in a Batch Ultrafiltration System

	Pure vancomycin		Fermentation filtrate	
Steps	μg	%	μg	%
Adsorption	258	100	165	100
Washing				
1	50	19.4	44	26.7
2	0	0	0	0
Elution	180	70.2	94	57.0
Total	230	89.6	138	83.7
Lost	28	10.4	27	16.3

macroligand is estimated to be ΔH = 14,000 cal/mol. The vancomycin binding to the macroligand is thus an endothermic process. However, the binding between vancomycin and diacetyl-L-lysine-D-alanyl-D-alanine studied by Nieto and Perkins (*14*) was an exothermic process. A possible explanation for the endothermic behavior of vancomycin binding to the macroligand is caused by the reduction of steric hindrance as the temperature is raised. Since the coiled macroligand molecule becomes more flexible and has a relatively open structure as temperature increases such that vancomycin is easier of access to the ligand D-alanyl-D-alanine.

Vancomycin Purification from Fermentation Liquor

Macroligand prepared by coupling agent carbonyldiimidazole was employed to purify vancomycin from fermentation liquor. The purification was carried out as a batch mode in Centricon 30. HPLC was used to analyze the samples from each purification step. As shown in the HPLC chromatograms of Fig. 4, the amount of vancomycin in the filtrate decreases after adsorption, which indicates the binding of vancomycin to the macroligand. However, most of vancomycin was lost in the filtrate at the adsorption step. This is because not enough macroligand was employed in the operation. During the first washing step, a small amount of vancomycin was washed out together with some contaminants. No vancomycin was detected during the second washing. Subsequently, the bound vancomycin was recovered upon elution with 0.5*M* ammonium solution of pH 11. The binding capacity of the macroligand is very stable. There is no appreciable decrease over six successive purification experiments. Table 3 summarizes the results of vancomycin purification from fermentation liquor and from pure vancomycin solution. The recovery yields of these two cases based on bound vancomycin are 57 and 70%, respectively. The low binding amount and high washing loss for fermentation liquor probably result from the high salts content in the liquor, which may affect the bind-

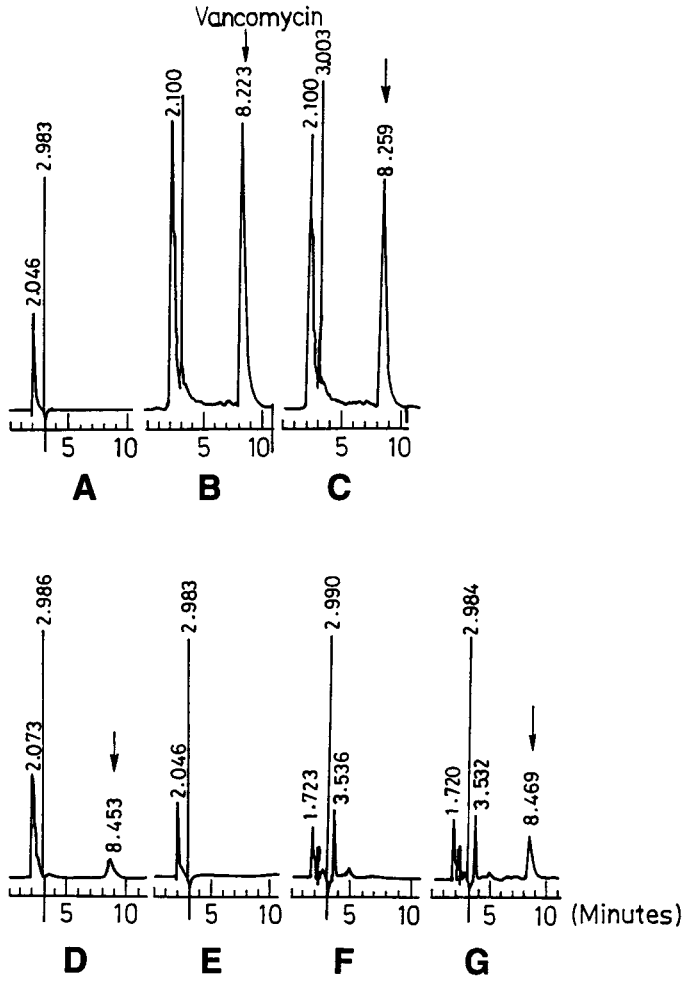

Fig. 4. HPLC chromatograms during vancomycin purification from fermentation liquor. (A) Phosphate buffer, (B) fermentation liquor before affinity ultrafiltration, (C) filtrate after mixing fermentation liquor with macroligand, (D) filtrate of first washing with phosphate buffer, (E) filtrate of second washing, (F) ammonium solution, and (G) filtrate of elution with ammonium solution.

ing interactions between ligand and vancomycin. The loss of vancomycin in the recovery process is probably owing to degradation during the elution step, since vancomycin is unstable at alkaline condition (*15*).

CONCLUSION

D-alanyl-D-alanine, the ligand for glycopeptide antibiotic vancomycin, has been coupled to dextran as a macroligand. The microligand retains the capacity to bind vancomycin and proves to be useful for purification

of vancomycin from fermentation liquor in affinity ultrafiltration. Vancomycin binding for the macroligand follows Langmuir type adsorption, as indicated by the equilibrium binding study. The binding characteristics are very different from those of binding between vancomycin and D-alanyl-D-alanine studied by Nieto and Perkins (*14*). The association constant is two orders of magnitude smaller and binding is an endothermic process. These may be attributed to the ligand attachment to the water soluble macromolecule without using the spacer arm. The steric hindrance limits the accessibility of vancomycin to the ligand. As temperature increases, the structure of coiled macroligand molecule becomes relatively open such that it allows more vancomycin to be bound with ligands.

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