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Synthesis and Characterization of D-Alanyl-D-Alanine-Agarose

A New Bioselective Adsorbent for Affinity Chromatography of Glycopeptide Antibiotics

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ABSTRACT

A new affinity adsorbent, using D-alanyl-D-alanine as ligand, has been prepared. The dipeptide immobilized on Activated CH-Sepharose 4B (D-Ala-D-Ala- AGA) bioselectively binds the glycopeptide antibiotics teicoplanin, vancomycin, ristocetin A (vancomycin-like group of antibiotics) while it does not bind other antibiotics equally active on cell wall biosynthesis but with different target sites, such as penicillin G, cephalosporin C, gardimycin, and bacitracin. Teicoplanin, vancomycin, and ristocetin A have similar binding characteristics for the immobilized dipeptide, as indicated by equilibrium binding experiments. The affinity constants of the three antibiotics for D-Ala-D-Ala-AGA is of the same order of magnitude (10^5 L mol^{-1}) and the number of effective binding sites is similar for each antibiotic (6–7 μEq/mL of gel). The adsorption is biospecific as no binding has been observed to immobilized L-alanyl-L -alanine.

D-Ala-D-Ala-AGA has been successfully used to purify teicoplanin from mixtures of different complexity and for concomitant extraction and purification from fermentation liquors by both batch adsorption and column chromatography. The antibiotic can be recovered from the resin in high yields by elution at pH 11.

Index Entries: Immobilized D-alanyl-D-alanine, bioselective adsorption of glycopeptide antibiotics on; bioselective adsorbent, immo-

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bilized D-alanyl-D -alanine as; affinity chromatography, glycopeptide antibiotic purification by; glycopeptide antibiotic extraction; affinity constants; equilibrium binding experiments.

INTRODUCTION

The yield of antibiotic production by microorganisms is usually low at the early stages of the discovery process and product development. Strain improvement is usually attempted at later stages when the therapeutic and commercial values of a new molecule have been assessed and the future development of the product outlined. Thus, identification, determination of novelty, structural studies, and the biological evaluation of a new antibiotic are usually made on the limited amount of material that can be produced by wild-type organisms. Consequently, it is important to have a quick and efficient method for extraction and purification from fermentation liquors early in the development process.

To this end, all extraction and purification technologies have been employed with the exception of affinity chromatography. This is surprising in view of the proven utility of affinity chromatography for the specific purification of biological molecules.

The possibility of developing a bioselective adsorbent for antibiotics was foreseen when it was found that the antibiotic teicoplanin (*1*), under development at our company, like other antibiotics of the so-called vancomycin-like group, inhibits cell wall synthesis by specific binding to the pentapeptide precursor of the cell wall that terminates with a D-alanyl-D-alanine residue at the carbonyl terminus (UDP-*N*-acetyl-muramyl pentapeptide) (*2,3*). Thus we have immobilized the dipeptide D-alanyl-D-alanine to an insoluble carrier and used it in the purification of teicoplanin from fermentation broths or from crude mixtures. Specificity of the affinity resin and association constants for a series of antibiotics of this glycopeptide class have also been determined. The affinity resin proves to be a powerful tool for the purification, identification, and selection of antibiotics that inhibits the bacterial cell wall synthesis by binding to the D-alanyl-D-alanine residue of the peptidoglycan chain.

MATERIAL AND METHODS

Chemicals

Activated CH-Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); D-alanyl-D-alanine, L-alanyl-L-alanine, and bacitracin Pharm. U.S.P. XVII were purchased from Serva Feinbiochemica (Heidelberg, Germany); vancomycin-HCl was from Eli Lilly (Indianapolis, USA); ristocetin A, penicillin G, and cephalosporin C were from Sigma Chemical Co. (St. Louis, USA), and gardimycin and

teicoplanin were prepared by Lepetit S.p.A. (Milano, Italy). Teicoplanin is a mixture of five closely related components (T-A_2 complex, formerly named teichomycin A_2) of similar polarity and a more polar product, T-A_3 (10% by weight of total complex). All other chemicals were analytical grade from Merck or Carlo Erba.

Synthesis of D-Alanyl-D-alanine-Agarose and L-Alanyl- L-alanine-Agarose

D -Alanyl-D-alanine and L-alanyl-L-alanine were coupled to activated CH-Sepharose 4B according to the manufacturer's instructions. A 30 mg portion of D-alanyl-D-alanine or L-alanyl-L-alanine, dissolved in 5 mL of 0.5*M* NaCl, 0.1*M* $NaHCO_3$ buffer at pH 8 was coupled in 1 h to 3 mL of gel. The unreacted ester groups were blocked with 1*M* ethanolamine-HCl at pH 9 for 1 h. The products were repetedly washed on a glass filter funnel with 0.5*M* NaCl, 0.1*M* Na-acetate buffer pH 4 and with 0.5*M* NaCl, 0.1*M* Tris-HCl buffer, pH 8. The resins obtained were named D-Ala-D-Ala-AGA and L-Ala-L-Ala-AGA.

Binding of Different Antibiotics to D-Ala-D-Ala-AGA and L-Ala-L-Ala-AGA

Equilibrium binding experiments with various antibiotics and D-Ala-D-Ala-AGA or L-Ala-L-Ala-AGA were carried out by a modification of the method of Tsapis et al. (*4*).

Fixed volumes (200 μL) of D-Ala-D-Ala-AGA or L-Ala-L-Ala-AGA suspension (1:10) in 0.15*M* NaCl, 0.05*M* Na-phosphate buffer, pH 7.4, were added to increasing concentrations of antibiotic in the same buffer (2 mL) and rotated end-over-end for 1 h at room temperature. All the samples were centrifuged for 5 min at 2000 rpm (Hereaus Christ Labofuge G 2) and the concentration of unbound antibiotic in the supernatants determined by optical density measurements. The bound material was calculated by subtracting the unbound from the total. All b inding data were elaborated according to Scatchard (*5*).

Affinity Chromatography of Teicoplanin

Affinity chromatography of a fermentation liquor containing teicoplanin (10 mL) was carried out on a D-Ala-D-Ala-AGA column (1.6 × 1.2 cm) preequilibrated in 0.15*M* NaCl, 0.05*M* Na-phosphate buffer, pH 7.4 (flow rate = 6 mL/h). After loading, the column was washed with the equilibration buffer and eluted with 0.15*M* NaCl, 0.05*M* Na-phosphate pH 11. The separation was monitored by transmittance at 280 nm. Peak fractions were pooled and immediately neutralized with HCl. The teicoplanin content of each fraction was estimated by HPLC analysis.

Salts were eliminated by ultrafiltration and extensive washings with distilled water on a Millipore PSAC 09005 (NMWL 1000) membrane. Solutions were lyophilized and tested for purity.

RESULTS AND DISCUSSION

Agarose was selected as the support matrix to immobilize D-alanyl-D-alanine in these studies. Perkins (*6*) made the observation that the minimum requirement for the complex formation between oligopeptides analogs of biosynthetic precursors of bacterial peptidoglycan and vancomycin is D-alanyl-D-alanine with the free terminal carboxyl group and the acylated *N*-terminus. According to this the dipeptide was coupled to activated CH-Sepharose 4B through the formation of a stable peptide bond between the six-carbon spacer arm of the support and the amino group of the dipeptide. The immobilized ligand is depicted in Fig. 1.

Selectivity of Binding of D-Ala-D-Ala-AGA

The resin was challanged for specificity versus glycopeptide antibiotics with a series of drugs with diverse mechanism of action on bacterial cell wall biosynthesis.

Teicoplanin, vancomycin (*7*), and ristocetin A (*7*) interfere with peptidoglycan synthesis by binding to the pentapeptide precursor having the D-alanyl-D-alanine residue at the carbonyl terminus. Penicillin G (*8*), cephalosporin C (*8*), gardimycin (*9*), and bacitracin (*10*) also interfere with the peptidoglycan synthesis too, but at different target sites. As expected, the resin was unable to bind penicillin G, cephalosporin C, gardimycin, or bacitracin, whereas it bound each of the vancomycin-like antibiotics.

Equilibrium Binding Experiments

Specificity of Binding

The adsorption of teicoplanin, vancomycin, and ristocetin A on D-Ala-D-Ala-AGA and on L-Ala-L-Ala-AGA was studied at various concentrations of antibiotic in order to evaluate the affinity and specificity for the immobilized dipeptides. The adsorption curves obtained by plotting the amount of antibiotic bound to the resin versus concentration (Fig. 2) show that the binding to immobilized D-alanyl-D-alanine occurs in the same concentration range (0.015–0.15 m*M*) for each drug. No binding to the immobilized L-isomer was observed. The absence of binding to L-Ala-L-Ala-AGA not only demonstrates the biological specificity of the affinity resin but also suggests that non specific ionic and hydrophobic interactions play a minor role.

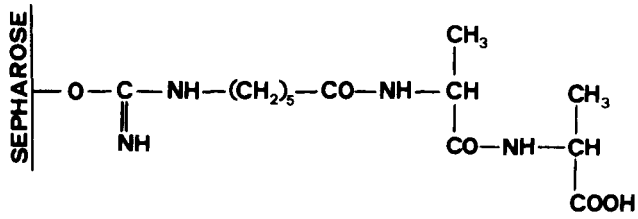

Fig. 1. Schematic representation of D-Ala-D-Ala-AGA.

The data of binding of each antibiotic, elaborated and plotted according to the method of Scatchard (insets, Fig. 2), resulted in linear plots, indicating binding to a single order of homogeneous combining sites. Thus unspecific binding caused, for example, by a large proportion of low affinity sites or by cooperative interactions with functional groups of the matrix, is irrelevant.

Binding Capacity

Repeated equilibrium binding experiments provided additional information on the maximal binding capacity of the gel for each antibiotic. The values of maximal binding capacity, indicated by the abscissa intercept in the Scatchard plot, are reported in Table 1. The maximal binding capacity of the adsorbent for the three different antibiotics is similar, suggesting that a comparable number of effective binding sites (functionally active D-alanyl-D-alanine) for each compound are available.

Affinity Constants

Brodelius and Mosbach (*11*) and Tsapis et al. (*4*) have shown that affinity chromatography can be properly used to determine the dissociation constants for binary complexes between a biomolecule and a specific ligand.

According to a modification of the method used by Tsapis et al. (*4*), we have determined the affinity constants (K_a) of teicoplanin, vancomycin, and ristocetin A for D-Ala-D-Ala-AGA. The affinity constants calculated as the negative slope of the Scatchard plot (insets, Fig. 2), are reported in Table 1. The affinity of the three antibiotics for the resin is of the same order of magnitude (10^5 L mol^{-1}). Moreover, the K_a of vancomycin is roughly similar to the association constants of this antibiotic for different D-alanyl-D-alanine terminating tripeptides as determined by Nieto and Perkins (*12*) by a different method.

The relative K_as of the three antibiotics determined in the present study are in agreement with those obtained by calorimetric measurements by Laynez (*13*) for *N*-acetyl-D-alanyl-D-alanine. In both cases the affinity constant of teicoplanin is 4–5 times that of vancomycin and 1–2 times that of ristocetin. However, the K_a values we obtained are higher than those obtained by Laynez. This should not be completely unexpected since the 6-carbon *N*-acyl chain provided by the spacer arm in the D-Ala-D-Ala-AGA may give a more effective contribution to the binding

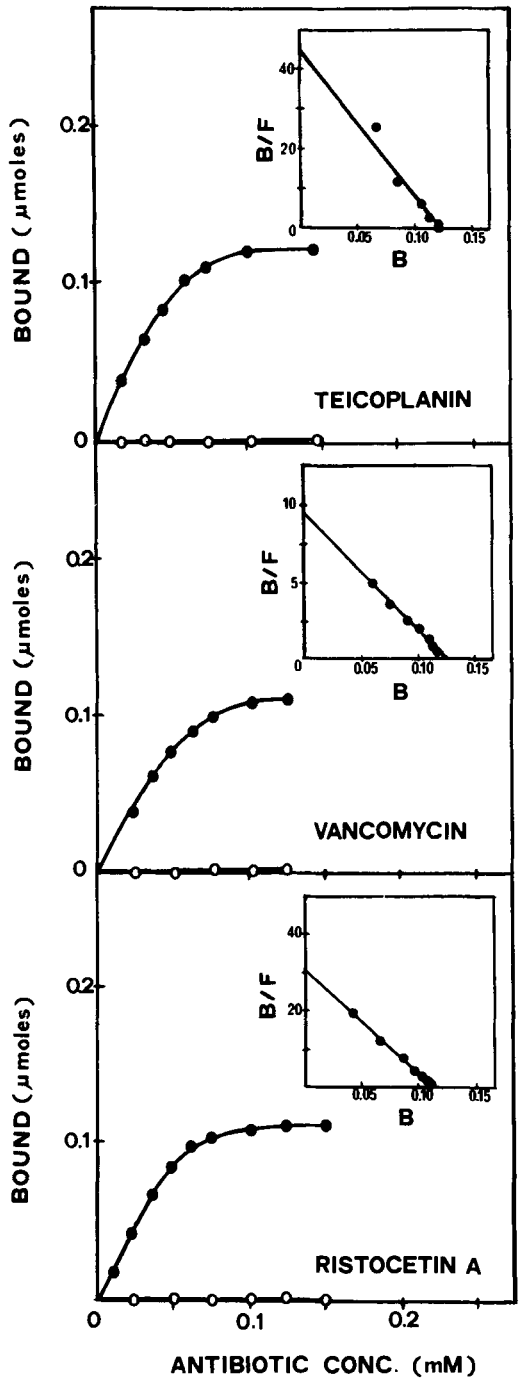

Fig. 2. Binding of teicoplanin, vancomycin, and ristocetin A to D-Ala-D-Ala-AGA (●) and to L-Ala-L-Ala-AGA (○) Insets: Scatchard plots.

than the acetyl group present in the dipeptide studied by Laynez. Indeed it has been shown by Nieto and Perkins (*12*) that a long aliphatic acyl

TABLE 1
Affinity Constants and Maximal Binding Capacity of DAla-DAla-AGA for Teicoplanin, Vancomycin, and Ristocetin A

Antibiotic	Maximal binding capacity,[a] μmol/mL of gel	Affinity constants,[a] L mol^{-1}
Teicoplanin	6.95 ± 0.46	(8.08 ± 0.38) × 10^5
Vancomycin	6.48 ± 0.20	(1.57 ± 0.21) × 10^5
Ristocetin A	6.05 ± 0.25	(3.89 ± 0.91) × 10^5

[a]Mean of three determinations ± SEM.

chain, such as myristic acid, on the *N*-terminus of D-alanyl-D-alanine improves the affinity of vancomycin for the dipeptide.

It seems reasonable to conclude that the affinity of the D-alanyl-D-alanine residue for the vancomycin-like group of antibiotics is not relevantly changed by immobilization on agarose.

Affinity Chromatography of Teicoplanin

The bioselective adsorption of teicoplanin on a D-Ala-D-Ala-AGA column was exploited in the separation of the antibiotic from fermentation liquors in which teicoplanin represents less then 5% of the dry material.

The separation of teicoplanin from other antibiotics or fermentation metabolites can easily be carried out by washing the column at pH 7.4 and by subsequent desorption at pH 11 (Fig. 3). About 94% of the loaded teicoplanin was recovered in the desorbed fraction (fraction II); the product was 65% pure. These data were constants over five experiments. The results clearly indicate that D-Ala-D-Ala-AGA can adsorb teicoplanin from crude preparation and that the antibiotic can be recovered in high yields and in a purer form. The resin has been succesfully used also for the batchwise purification and concentration of teicoplanin from biological fluids such as urine and plasma (data not shown).

CONCLUSION

D-Alanyl-D-alanine, the bacterial cell wall-binding site for glycopeptide antibiotics (vancomycin-like group) has been immobilized on agarose.†

The immobilized ligand retains the capacity to bind antibiotics selectively and proves to be a useful tool for the selection, extraction, and purification of glycopeptide antibiotics from fermentation liquors.

†Patent applications, covering D-Ala-D-Ala matrix, are pending in several countries.

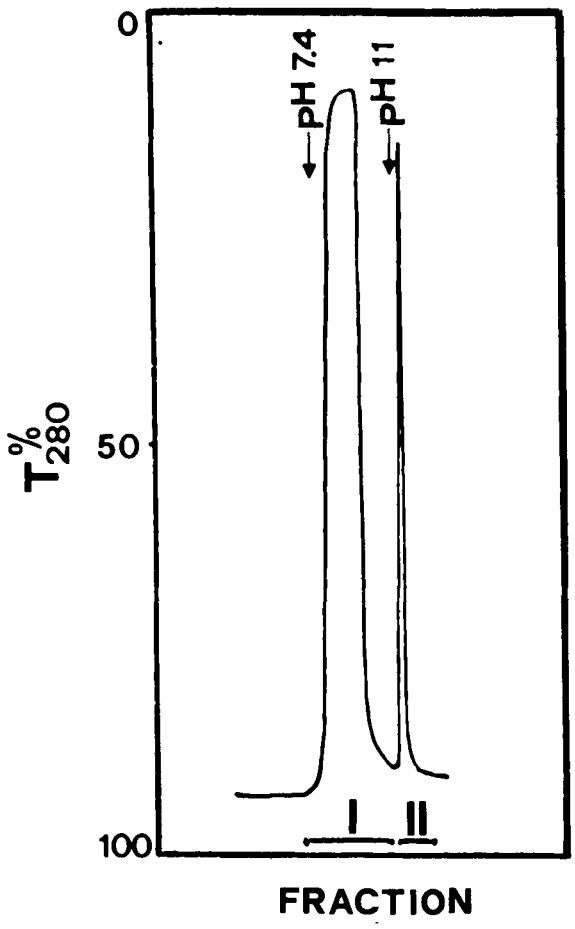

Fig. 3. Affinity chromatography on a D-Ala-D-Ala-AGA column of teicoplanin fermentation liquor.

Equilibrium binding studies have shown the matrix to be made of homogeneous combining sites that allow the determination of the affinity constants of each antibiotic for this specific immobilized biological target. Therefore the affinity matrix provides a useful model system for studying interaction of the cell wall component and antibiotics.

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