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AFFINITY CHROMATOGRAPHY OF GLYCOPEPTIDE ANTIBIOTICS

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SUMMARY

An affinity support was designed to facilitate the isolation and purification of glycopeptide antibiotics by mimicking their known affinity for the bacterial cell wall. Members of this class of antibiotics inhibit peptidoglycan biosynthesis by specifically binding to pentapeptide precursors terminating with L-Lys–D-Ala–D-Ala. A series of ligands (Gly, D-Ala, D-Ala–D-Ala and α-N-Ac–L-Lys–D-Ala–D-Ala) were immobilized on an N-hydroxysuccinimide-activated agarose support and evaluated using the glycopeptides vancomycin and the aridicin complex. Conditions were developed to enable complete adsorption and efficient elution of both antibiotics. Of the four ligands, the readily available dipeptide offered the best compromise between high binding specificity and recovery on elution. Binding and subsequent high recovery of biologically active products were observed for eight other glycopeptide antibiotics. Column performance was shown by purification of vancomycin directly from a low titer fermentation broth. The applicability of this technique to large scale isolation was demonstrated by the preparative affinity chromatography of 36 g of the aridicins.

INTRODUCTION

Vancomycin, a member of the glycopeptide class of antibiotics, is a clinically important therapeutic agent used for the treatment of infections due to methicillin resistant *Staphylococcus* sp.[1]. Over ten members of this class have been reported, all of which are structurally related to vancomycin (Fig. 1). As part of a screening program aimed at discovery of novel glycopeptides[2] we have developed an affinity support to specifically isolate and purify members of this class based on their ability to bind to peptides terminating in D-Ala–D-Ala. Affinity chromatography, more commonly used as a tool for protein purification[3,4], requires that the molecules to be isolated recognize or bind to a specific receptor or ligand. Almost all reported examples are those in which at least one of the interacting species is a protein or large peptide. However, examples where both the ligate and ligand are of low molecular

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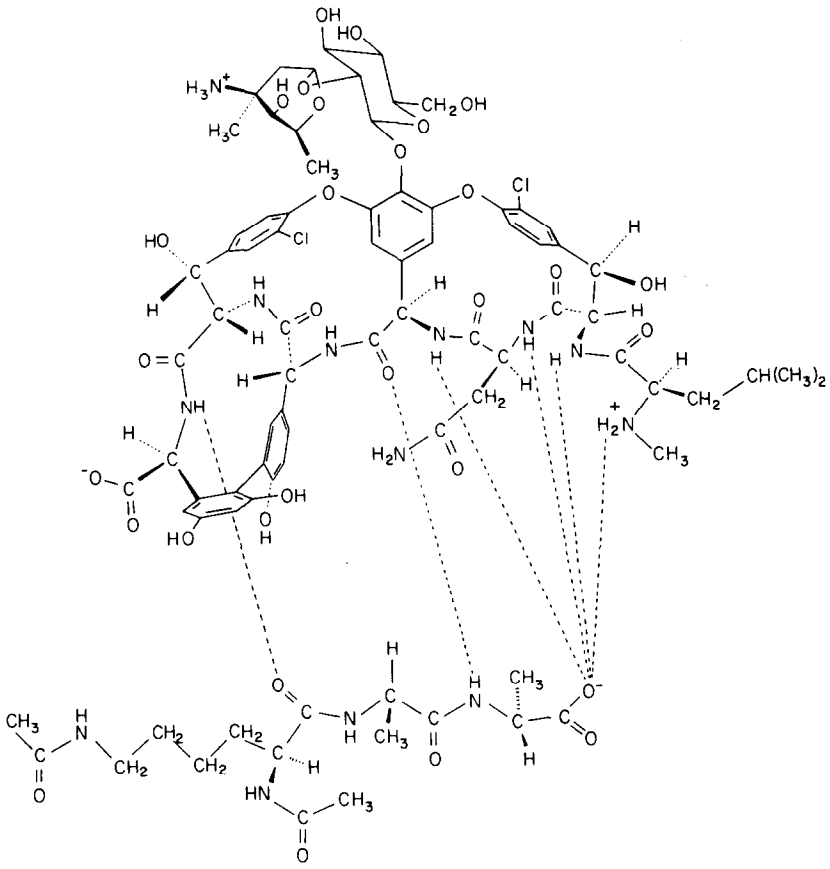

Fig. 1. Proposed interactions between vancomycin and Ac_2–L-Lys–D-Ala–D-Ala. Taken from Williams *et al.*[14].

weight are rare, limited at present to chiral columns[5]. The structures posed by two potentially interacting low-molecular-weight ligates have few sites available to form a tight binding complex. Glycopeptide antibiotics are an important exception to this since they inhibit peptidoglycan biosynthesis in the bacterial cell wall by specifically binding to pentapeptide precursors terminating with L-Lys–D-Ala–D-Ala[6,7] and are themselves less than 2000 molecular weight. The binding of these low-molecular-weight glycopeptides to cell wall precursors is analagous to that observed for protein–ligand interactions and suggests it can also be exploited using the technique of affinity chromatography.

The utility of an affinity support requires both a minimum of non-specific binding selectivity and an effective desorption procedure. Solution binding studies by Nieto and Perkins[8,9] have previously shown that the glycopeptide antibiotics vancomycin and ristocetin bind to the tripeptide Ac_2–L-Lys–D-Ala–D-Ala with a binding constant of 10^6 l mol^{-1} and to several dipeptide derivatives containing Ac–D-Ala–D-Ala with binding constants of 10^4–10^5 l mol^{-1}. Model studies of solid-state interactions performed with insoluble peptide made by linking α-N-Ac–L-Lys–D-Ala–D-

Ala to CM-cellulose demonstrated that adsorption of vancomycin mimics its natural binding to cell wall preparations[10]. Unfortunately, desorption of bound vancomycin from this support was difficult and recovery of more than 50% of the antibiotic was never achieved. Recently, Corti and Cassani[11] described an affinity technique for isolating small quantities of teicoplanin from fermentation broths on Sepharose 4B–D-Ala–D-Ala. A recovery of 90% of antibiotic was reported but the product purity was only 65%.

In this paper, we report our independent findings on the development of an affinity support for purification of glycopeptide antibiotics. This includes preparation and characterization of various immobilized ligands mimicking the natural pentapeptide and the development of high recovery elution conditions. The utility of the optimal matrix, Affi-gel 10–D-Ala–D-Ala, is described for small and large scale isolations of vancomycin and the aridicins[12,13].

EXPERIMENTAL

Materials

Ethanolamine, D-alanine, glycine, ristocetin and vancomycin were purchased from Sigma. D-Alanyl–D-alanine and L-alanyl–L-alanine were products of Vega Biochemicals. The antibiotic A35512B was provided by B. Abbott, Lilly Research Laboratories; avoparcin by D. Borders, Lederle Laboratories; and teicoplanin by M. R. Bardone, Gruppo Lepetit. Broths containing vancomycin (ATCC No. 19795) and the aridicins (ATCC No. 39323) were obtained by fermentation. The antibiotic OA-7653 (ATCC No. 31613) was obtained by fermentation. The tripeptide α-N-Ac–L-Lys–D-Ala–D-Ala was provided by Dr. M. Moore, Smith Kline and French Laboratories.

Methods

High-performance liquid chromatography (HPLC) assays for glycopeptide antibiotics. Quantitation of antibiotic concentrations was performed with a Beckman Model 344 liquid chromatograph using an Altex Ultrasphere ODS (150 × 4.6 mm I.D.) 5-μm column monitoring at 254 nm with a Beckman Model 160 detector. The column was equilibrated with 0.1 *M* phosphate buffer, pH 3.2 and then eluted with a linear gradient of 7–30% acetonitrile over 13 min at a flow-rate of 2 ml/min. Alternatively, isocratic systems were developed using 0.1 *M* phosphate buffer, pH 3.2 and 8, 9, 10, 15 or 25% acetonitrile for the antibiotics ristocetin, A35512B, avoparcin, OA-7653 and teicoplanin respectively.

Preparation of affinity supports. Immobilization of Affi-gel 10® (Bio-Rad Labs., Richmond, CA, U.S.A.) was carried out according to the manufacturers instructions. A 2-fold excess of ligand to active ester was used in the coupling reaction and unreacted active groups were blocked with 1 *M* ethanolamine adjusted to pH 9.

Determination of bound ligand. Affinity gel samples were hydrolyzed in 5.7 *M* hydrochloric acid at 110°C for 24 h and analyzed for bound ligand by amino acid analysis by Sequemat (Watertown, MA, U.S.A.).

Determination of maximum column capacity. A sample of the affinity gel was equilibrated in 0.02 *M* phosphate buffer at pH 7.0 and 0.5-ml aliquots were transferred to each of four tubes. A volume of 4 ml of a vancomycin solution containing

a 3-fold excess of antibiotic to immobilized ligand was added to each tube, the slurry mixed for 15 min at ambient temperature and centrifuged at 500 *g* for 10 min. The supernatant was removed and the gel washed four times with 4 ml of equilibration buffer. The amount of vancomycin bound was measured by subtractive HPLC analysis of the spent and washes.

Purification of glycopeptide antibiotics from microbial fermentation broths. Fermentation broths of glycopeptide producing organisms were clarified by centrifugation at 5000 *g* for 30 min at 4°C, the supernatant adjusted to pH 7.0 and filtered through a Whatman GF/D glass fiber filter. Clarified broth (10–20 ml) was loaded onto a 10 × 1 cm I.D. column containing 2 ml of Affi-gel 10–D-Ala–D-Ala previously equilibrated with 0.02 *M* sodium phosphate at pH 7.0. The gel was washed with 20 ml of equilibration buffer, 15 ml of 0.4 *M* ammonium acetate, pH 7.8 and 15 ml of 10% acetonitrile. Glycopeptide was eluted with 0.1 *M* ammonia containing 50% acetonitrile. Fractions of 1 ml were collected at a flow-rate of 2 ml/min and monitored for product by absorbance at 280 nm and HPLC analysis. The affinity purified product was recovered by lyophilization. The gel was regenerated by washing with 20 column volumes of 0.4 *M* sodium carbonate pH 9.5 buffer containing 30% acetonitrile and reequilibrated with 0.02 *M* sodium phosphate at pH 7.0.

Preparative affinity chromatography of the aridicin complex. Preliminary isolation of the aridicin complex from 600 l of fermentation broth was carried out as previously described[13] (Fig. 2). The XAD-7 concentrate was adjusted to pH 7 and purified in three batches on a 1-l column of Affi-gel 10–D-Ala–D-Ala. The column

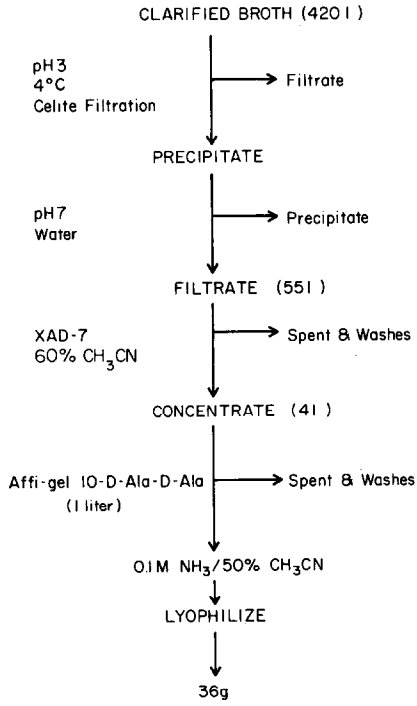

Fig. 2. Large-scale isolation of the aridicins.

was washed with 2 l of 0.02 *M* sodium phosphate, pH 7, 2 l of 1% acetonitrile and eluted with 4 l of 0.1 *M* ammonia containing 50% acetonitrile. The three pools of affinity purified aridicins were combined and lyophilized.

RESULTS AND DISCUSSION

Selection of ligands and support

The choice of ligands used in this study was based on the original solution binding studies carried out on the glycopeptides vancomycin and ristocetin B by Perkins and Nieto[8,9]. A portion of their results are outlined in Table I. The strongest binding was observed for the tripeptide, Ac_2–L-Lys–D-Ala–D-Ala, with almost 10-fold lower affinity observed for the dipeptide Ac–D-Ala–D-Ala. In all cases, a D-configuration is required in both alanines for binding to occur. Longer peptides approaching the length of the natural pentapeptide (L-Ala–D-γ-Glu–L-Lys–D-Ala–D-Ala) failed to exhibit tighter binding. More recent studies by Williams and co-workers[14–17] using NMR techniques have led to the proposed model shown in Fig. 1 for the interactions of vancomycin with the tripeptide Ac_2–L-Lys–D-Ala–D-Ala. Although several of the ligands listed in Table I effectively mimicked the natural pentapeptide with binding constants in excess of 10^4 l mol^{-1}, studies were undertaken to see if the tripeptide sequence was required for effective purification of glycopeptide antibiotics or if a smaller fragment would suffice. The ligands selected for immobilization are listed in Table II. They include the tripeptide having a free ε-amino group plus shorter segments such as the dipeptide D-Ala–D-Ala and D-Ala alone (both with free α-amino groups as attachment sites). Although gels of high ligand density would appear to be advantageous to obtain high loading capacity, low recovery is often observed for macromolecules on high-capacity supports. To study this, gels of high and low ligand density were prepared by varying the ratio of ligand to activated sites on the support in the reaction mixture. A commercially available activated support containing a 10 carbon spacer terminating in an N-hydroxysuccinimide ester, Affi-gel 10, was used for these studies. Following immobilization of the tripeptide and D-Ala–D-Ala dipeptide ligands at various coupling ratios, the expected corresponding

TABLE I

BINDING CONSTANTS IN SOLUTION

Data taken from Nieto and Perkins[8,9].

	K_A ($l\ mol^{-1}$)	
	Vancomycin	*Ristocetin B*
Ac_2–L-Lys–D-Ala–D-Ala (D) (L)	$1.5 \cdot 10^6$	$5.9 \cdot 10^5$
Ac_2–L-Lys–(L)-Ala–(D)-Ala (L) (L)	0	0
Ac–D-Ala–D-Ala	$2.0 \cdot 10^4$	$7.2 \cdot 10^4$
Ac–Gly–D-Ala	$1.1 \cdot 10^4$	$4.9 \cdot 10^4$
Myristoyl–D-Ala–D-Ala	$5.9 \cdot 10^4$	–

TABLE II

LIGANDS AND BINDING CAPACITIES

Ligand	*Coupling* ratio*	*Coupled** ligand (μmol/ml)*	*Capacity for *** vancomycin (μmol/ml)*	*Capacity/ coupled ligand*
α-Ac-L-Lys-D-Ala-D-Ala	0.3	1.5	2.1	1.4
	2.3	8.2	13	1.6
D-Ala-D-Ala	0.3	1.6	2.0	1.3
	1.0	3.9	6.2	1.6
	2.0	5.5	8.8	1.6
L-Ala-L-Ala	2.0	4.3	0	–
D-Ala	2.0	8.0	11	1.4
Gly	2.0	4.6	7.7	1.7
Ethanolamine	2.0	–	0	–

* μmol ligand added per μmol activated sites on gel. Maximum sites equal 15 μmol/ml gel.
** Measured by amino acid analysis of acid hydrolyzed gel samples.
*** Amount of vancomycin bound per ml gel at pH 7; measured by UV.

increases in the amount of coupled ligands as measured by amino acid analyses were observed (Table II).

Vancomycin bound to all immobilized ligands terminating in D-Ala (Table II). Antibiotic did not bind to a control gel which was completely blocked by reaction with ethanolamine, suggesting there are no non-specific interactions between vancomycin and the Affi-gel 10 backbone, spacer arm or blocking ligand. As predicted by Perkins and Nieto (Table I), binding is lost when D-Ala-D-Ala is substituted by L-Ala-L-Ala. This implies that immobilized L-Ala-L-Ala did not function as an effective ion-exchange support under the conditions studied even though vancomycin is positively charged at neutral pH [p*I* 8.1 (ref. 18)] and the gel is negatively charged. Vancomycin did bind to immobilized Gly. As will be shown later, this could be readily reversed by increasing the ionic strength, suggesting an ion-exchange type of interaction.

A common problem encountered during affinity purification of proteins is their inaccessibility to densely packed ligand because of steric hindrance. Because glycopeptide antibiotics have relatively low molecular weights (1500–2000) compared to typical proteins (> 10 000), the capacity would be expected to match the ligand density (assuming a 1:1 ratio of antibiotic to ligand). This property was examined by measuring the maximum amount of vancomycin bound to each of the prepared supports. In each case, the capacity exceeded the amount of bound ligand by factors of 1.3–1.7 (Table II). This property is independent of the immobilized ligand and increases with the amount of bound ligand on the gel. Excess binding capacity was also observed by other workers[19] and may be attributed to the tendency of these antibiotics to aggregate.

Although each of the prepared supports containing one or more amino acid components of the natural ligand (as well as the support containing glycine) bound vancomycin, the performance of each gel was further evaluated in terms of product recovery. Binding interactions for glycopeptide antibiotics are believed to involve ionic, hydrogen bonding and hydrophobic interactions[14,20] which are illustrated in

Fig. 1. Elution of vancomycin from the tripeptide, D-Ala and Gly supports was studied with particular emphasis on disruption of ionic and hydrophobic interactions as listed in Table III. Solution studies predict (Table I) that the binding constants of the immobilized ligands should increase going from Gly to D-Ala to α-N-Ac-L-Lys-D-Ala-D-Ala. As expected, elution of vancomycin by disruption of ionic forces alone followed this trend (Table III). At pH 8, near the isoelectric point of vancomycin, 66% of bound antibiotic was eluted from the Gly support, 29% was recovered from the dipeptide support and no antibiotic was eluted from the tripeptide support with 0.5 *M* sodium chloride. Using a pH 9.5 buffer (0.5 *M* sodium carbonate), which is above the isoelectric point of vancomycin, almost full recovery could be obtained from the immobilized Gly. The same conditions yielded only 61% recovery from the dipeptide gel. Since a further increase in pH could lead to air oxidation or degradation of the antibiotic, other means of elution by complex disruption were evaluated.

The use of acetonitrile–water mixtures to disrupt hydrophobic interactions without pH or ionic strength adjustments (Table III) showed poor recoveries from all of the supports. Except for the case of the tripeptide support, full recovery of vancomycin was achieved by elution with both high pH plus acetonitrile. These results imply that in the solid state there are hydrophobic as well as ionic interactions between vancomycin and both the immobilized tripeptide and dipeptide ligands. Similar, but weaker, interactions are present with D-Ala alone. The Gly support displays minimal hydrophobic interactions and operates primarily as an ion exchange gel.

The poor recovery (40%) of tightly bound antibiotic from the tripeptide gel with the combined solvent system could be overcome by lowering the ligand density at the expense of overall capacity. The recovery of vancomycin increased to 92% using 0.1 *M* ammonia–50% acetonitrile elution from gel prepared at a density of 1.5 μmol/ml (Table II). Further studies showed that because of the tighter binding to the immobilized high density tripeptide, elution volumes were greater than those observed with equivalent volumes of either low- or high-density dipeptide supports. The

TABLE III

DEVELOPMENT OF ELUTION CONDITIONS FOR HIGH-CAPACITY GEL

A solution containing 16 mg vancomycin in 0.02 *M* phosphate, pH 7, was batched onto 0.5 ml of each support and eluted with 4 × 4 ml of each eluent. Vancomycin was determined by UV.

Interaction	*Eluent*	*Recovery (%)*			
		Gly	D-*Ala*	D-*Ala*–D-*Ala*	α-*Ac*-L-*Lys*-D-*Ala*-D-*Ala*
Ionic	0.5 *M* NaCl, pH 8	66	38	29	0
	0.4 *M* Carbonate, pH 9.5	82	73	61	–
Hydrophobic	Acetonitrile–water (30:70)	27	20	23	–
Ionic + hydrophobic	0.4 *M* sodium carbonate, pH 9.5–acetonitrile (70:30)	104	100	100	40
	0.1 *M* ammonia–acetonitrile (50:50)	100	100	100	–

TABLE IV

MODIFICATION OF ELUTION CONDITIONS FOR PURIFICATION FROM FERMENTATION BROTHS

Fermentation broth containing 8 mg of antibiotic was loaded onto 2 ml of each support and the gels sequentially eluted with 10 ml of each buffer or solvent. Recoveries were measured by HPLC.

Antibiotic	*Isoelectric point*	*Ligand*	*Recovery (%) on sequential elution*			
			0.02 M Phosphate pH 7	*0.05 M Ammonium acetate pH 7.8*	*Acetonitrile–water (10:90)*	*0.1 M Ammonia–acetonitrile (50:50)*
Vancomycin	8.2	Gly	32	59	8	0
		D-Ala	0	15	4	81
		D-Ala–D-Ala	0	5	0	95
Aridicin complex	3.8	Gly	84	5	11	0
		D-Ala	7	2	85	6
		D-Ala–D-Ala	1	0	72	27

tripeptide gel required approximately double the eluting solvent needed for the dipeptide support due to tailing and slightly later elution of the bound vancomycin. Although the low-density tripeptide gel could be used for purification, its use as a routine support was considered undesirable for several reasons: it is expensive to prepare because the ligand is not commercially available; it has a low loading capacity; and it requires large elution volumes.

Because of the disadvantage of the tripeptide gel, studies were undertaken to determine if binding of vancomycin and other glycopeptide antibiotics to the other gels would be tight enough to allow the removal of non-specifically bound contaminants present in a crude fermentation broth prior to elution of pure product. The results on affinity chromatography of a crude broth sample were similar to those previously observed for elution of the pure standard. Table IV shows the amounts of vancomycin sequentially eluted by each eluent. The bulk of the vancomycin was eluted from the Gly gel in the low and high ionic strength washes along with adsorbed colored broth contaminants. In contrast, recovery of purified antibiotic from the D-Ala gel increased to 81% in the ammonia–acetonitrile elution but 19% bled off the column in an attempt to remove weakly bound contaminants with the prewashes. The dipeptide gel had the greatest specificity in that 95% of the bound vancomycin was recovered in the final eluant and only 5% was lost in the prewashing steps. Because removal of broth contaminants requires both high ionic strength (0.5 *M* acetate) and organic solvent (acetonitrile) prewashes, the D-Ala–D-Ala ligand was the smallest peptide of those studied which maintained sufficient selectivity between non-specifically adsorbed materials and the antibiotic.

To test the generality of this observation, the same series of experiments were repeated using the aridicin complex since these antibiotics show a significant variation in chemical properties as compared to vancomycin. The aridicins (USAN ardacin) are novel glycopeptide antibiotics which differ from vancomycin in that they contain C_{10} to C_{12} lipid side-chains and a carboxyl group instead of an amino group on their carbohydrate appendages giving them a net negative charge at neutral pH[12,13]. The difference in isoelectric points between vancomycin and the aridicin complex [8.1 *vs.* 3.8 (ref. 18)] offered a good test as to whether the negatively charged complex could be purified on a negatively charged matrix. The Gly, D-Ala and dipeptide gels were retested with the aridicin complex and the results are presented in Table IV. As anticipated, the Gly support was ineffective with the bulk of antibiotics appearing in the early washes, most likely due to charge repulsion. For the D-Ala containing gels, an additional interaction was evident in that minimal breakthrough of the complex occurred at pH 7 in spite of net negative charge on both the matrix and the antibiotic. Since over 70% of the aridicin complex could be recovered by elution with acetonitrile–water (10:90) for both gels, the extra interaction was presumably hydrophobic in nature. Although the leakage of the antibiotic complex from the dipeptide gel was substantial, effective purification could be achieved by prewashing with one column volume of acetonitrile–water (1:99) to remove contaminants and then eluting bound complex with 0.1 *M* ammonia–acetonitrile (50:50) in high recovery (see the large-scale isolation described below).

The general utility of the D-Ala–D-Ala ligand was investigated by examining binding and elution for a series of other glycopeptide antibiotics including ristocetin[16,17], A35512B[21], avoparcin[22], OA-7653[23] and teicoplanin[24] (Table V). Since we

TABLE V

BINDING AND RECOVERY OF GLYCOPEPTIDE ANTIBIOTICS FROM AFFI-GEL 10–D-Ala–D-Ala

Antibiotic was loaded onto 1 ml of affinity gel at pH 7 and eluted with 5 ml of 0.1 *M* ammonia–acetonitrile (50:50). Recoveries were measured by HPLC.

Glycopeptides	*mg applied/ml gel*	*Bound (%)*	*Recovered (%)*
Vancomycin	0.1	100	84
Avoparcin	0.1	100	66
A35512B	1.4	100	85
OA-7653	0.1	100	70
Ristocetin	4.6	100	83
Teicoplanin	0.1	100	70
Aridicin complex	2.5	100	84

were limited by the small amounts of some of these standards, the binding and recovery of vancomycin was reexamined at concentrations falling within the range of available standard materials. The observed 84% recovery of vancomycin at the 0.1-mg level was considered optimal. Compared to this, the recoveries (66–85%) seen with the reference glycopeptides were reasonable. Because binding and high-recovery elution were both easily achieved for each of the glycopeptide antibiotics studied here, the D-Ala–D-Ala affinity support could be predicted to serve as a generic affinity support for isolating similar glycopeptide antibiotics operating by the same mechanism. The class-specific binding and elution became the basis for the use of this gel in our glycopeptide screen[2] to efficiently isolate and purify any member of this class encountered including novel entities previously unreported.

Applications

Since this simple 1-step chromatographic scheme was designed to generically purify all glycopeptide antibiotics directly from fermentation broths, its success as a screening tool depended on the ability of the immobilized dipeptide to capture antibiotics present at low titers in extremely complex fermentation milieus. The performance of the D-Ala–D-Ala affinity gel was tested for its efficiency and specificity by loading 10 ml of clarified broth containing 0.02 mg/ml vancomycin and approximately 20 mg/ml of other solids directly onto 2 ml of gel. The elution profile of vancomycin from the affinity column is illustrated in Fig. 3. The major peak of activity occurs following the high pH–acetonitrile elution. Fig. 4A shows the analytical HPLC profile of the initial broth sample. The concentration of vancomycin is near the detection limit of the HPLC method and its peak can barely be seen. Fig. 4B is the corresponding chromatogram of the purified antibiotic (retention time 5.5 min) recovered by lyophilization of the ammonia–acetonitrile wash and reconstitution in a smaller volume. The major peak in this chromatogram exhibited co-retention with authentic vancomycin while the minor peak (retention time 8 min) co-eluted with vancomycin aglycone prepared separately[25]. In general, sugar residues vary in both number and type for this class of antibiotics and play only a secondary role in

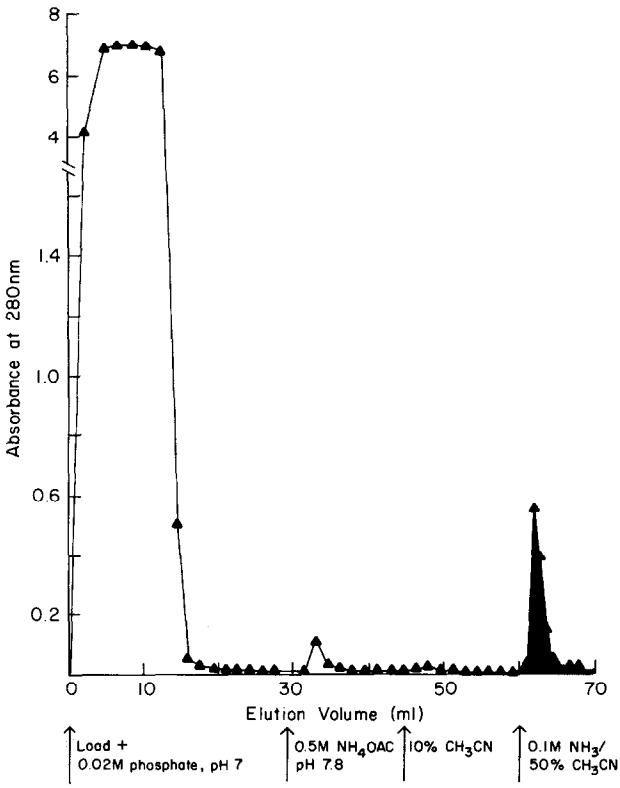

Fig. 3. Affinity purification of vancomycin from clarified fermentation broth. Clarified broth (10 ml) was chromatographed on a 10 × 1 cm I.D. column containing 2 ml of Affi-gel 10–D-Ala–D-Ala. The effluent was monitored by UV at 280 nm. Elution of vancomycin is indicated by the shaded region. NH_4OAC = Ammonium acetate.

the specific receptor interactions[6,7,26]. Therefore, co-purification of the aglycone would be expected. The purification of vancomycin was independent of the broth titer, since highly purified antibiotic could be recovered from a low-titer broth. Vancomycin purified by this procedure is biologically and structurally [UV, IR, fast atom bombardment mass spectrometry (FAB-MS) and NMR] identical to an authentic standard.

The scale-up potential of the dipeptide support was demonstrated by preparative affinity purification of the aridicin complex from 600 l of fermentation broth (Fig. 2). Two modifications of the small scale studies were incorporated into the final large-scale process. An initial precipitation and a chromatography step on XAD-7 were added to reduce the initial 600-l volume down to 4 l and to remove broth contaminants which tended to foul the affinity gel. Secondly, after the XAD-7 concentrate was loaded onto the affinity gel, the column was washed with both 0.5 *M* ammonium acetate, pH 7.8 and 1% acetonitrile prior to elution with 0.1 *M* ammonia containing 50% acetonitrile. This acetonitrile prewash step using 1% instead of 10% solvent resulted in less than 5% loss of bound aridicins and eliminated the bulk of colored contaminants. The 36 g of purified product obtained after direct lyophili-

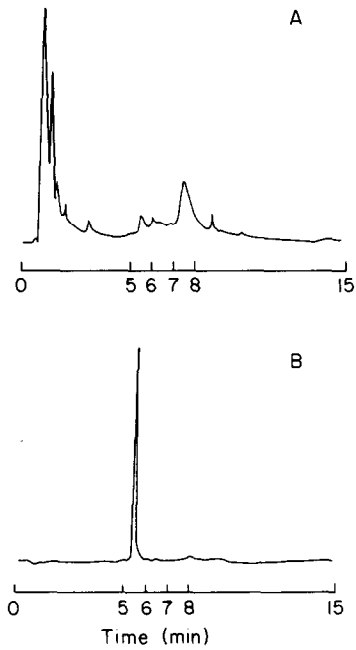

Fig. 4. Analytical HPLC analysis of affinity purified vancomycin. In each case, 20-μl samples were injected onto a 150 × 4.6 mm I.D. Altex Ultrasphere ODS 5-μm column previously equilibrated with 0.1 *M* potassium phosphate, pH 3.2. Bound components were eluted with a 7–30% acetonitrile gradient over 13 min at a flow-rate of 2 ml/min. The effluent was monitored for UV absorbance at 254 nm. (A) Starting broth, 0.2 a.u.f.s. (B) Affinity purified vancomycin, 0.1 a.u.f.s.

zation was identical in purity to the 8-mg sample purified in the small-scale experiment. The product was a white powder which contained less than 1% inorganic material on combustion analysis. The analytical HPLC of this product (Fig. 5) shows at least 25 peaks all of which were later identified as aridicin-like glycopeptides. The components were found to vary only in their glycolipid moieties[27] as determined by their UV analyses, isoelectric points, FAB-MS, and carbohydrate and lipid compositions. The major components, aridicins A, B and C, were identical to those obtained by conventional chromatography[13].

Affinity chromatography with Affi-gel 10–D-Ala–D-Ala can be used either in a batch-type procedure with a 1:5 or 1:10 volume ratio of gel to sample or by direct flow through a column. The kinetics of binding at room temperature were fast with quantitative binding of antibiotic at flow-rates up to 120 cm h^{-1} until the capacity of the column was reached. Similarly, a 10–15 min contact time in batch mode was sufficient. Binding occurred under a variety of conditions typically encountered in fermentation broths and was not sensitive to batch to batch variations. All glycopeptides we have examined bind to the gel at neutral pH. For those not binding as tightly, such as the aridicin complex, selective desorption is an effective means to discriminate glycopeptide antibiotics of varying charge[28,29]. Several elution conditions were examined for vancomycin and none were superior to the 0.1 *M* ammonia–acetonitrile (50:50) mixture. It offered both high recovery of bound anti-

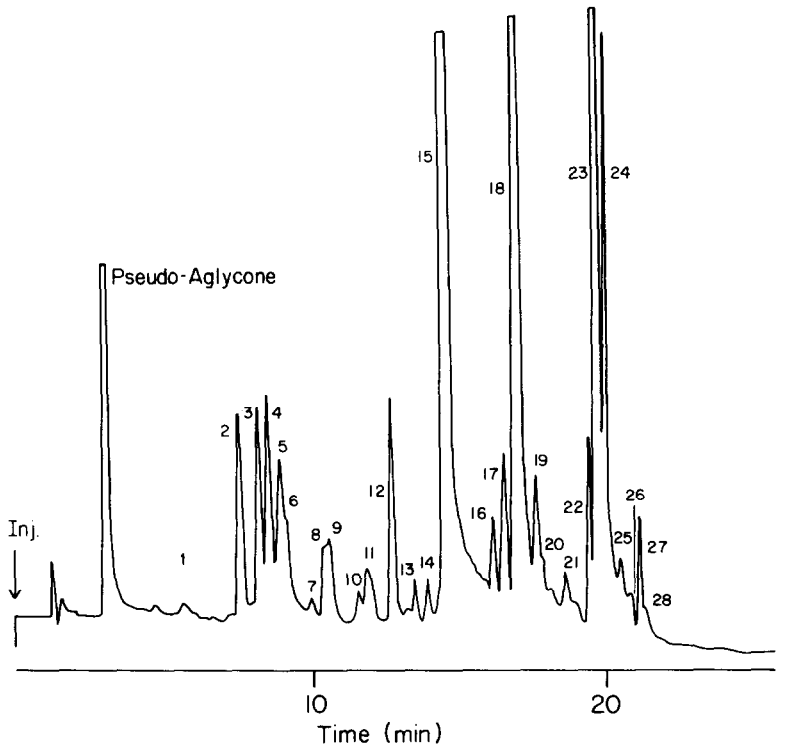

Fig. 5. Analytical HPLC of affinity purified aridicins. Details of the chromatography are described under Fig. 4 with the exception of using a 25–60% gradient of acetonitrile over a 13-min interval (UV 220 nm at 0.5 a.u.f.s.).

biotic and the ability to isolate pure product by lyophilization without needing a subsequent chromatography step to remove non-volatile salt components. Replacement of acetonitrile by methanol or ethylene glycol or elution at pH values of 2.5–9.5 using 4 *M* sodium chloride without acetonitrile resulted in lower product recovery.

CONCLUSIONS

Although affinity chromatography is an established art in the field of protein chemistry, we have extended its use to low-molecular-weight molecules. The interaction of glycopeptide antibiotics and D-Ala containing ligands appears structurally selective. Although binding readily occurs to the dipeptide and tripeptide gels, specific conditions for high recovery of antibiotic had to be found. Our unique process involving 0.1 *M* ammonia containing 50% acetonitrile results in high recoveries of at least eight antibiotics of this class and has the added advantage that the recovery solvents can be easily removed from the product by lyophilization.

The 1-step purification process is now routinely used as both a rapid screening assay for new antibiotic producing cultures as well as a means for providing milligram quantities of potentially novel antibiotics for biological evaluation and structure studies. The selectivity and avidity of the capture step permits useful information to be

obtained with small fermentation samples, and when necessary, lends itself to large scale isolation procedures for purification of gram quantities of antibiotic.

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