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A peptide binding chromogenic assay for detecting glycopeptide antibiotics

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SUMMARY

A solid-phase peptide binding assay, based on the mechanism of action of glycopeptide antibiotics, was developed for detecting this chemical class of metabolites. Utilizing a pentapeptide (L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine)–bovine serum albumin conjugate immobilized on the wall of microtiter wells, the binding of the vancomycin–alkaline phosphatase to the peptide could be demonstrated by subsequently monitoring the enzyme activity. The presence of glycopeptides in fermentation broths could be detected and quantified with a competitive binding assay. Peptides with a D-alanyl-D-alanine carboxyl terminus were necessary for the binding of these glycopeptides, thus confirming the mode of action of this class of antibiotics.

INTRODUCTION

Vancomycin has emerged in the 1980s as an important clinical antibiotic for treating methicillin-resistant strains of *Staphylococcus aureus* and other gram-positive infections. This antibiotic belongs to a chemically related family of glycopeptides such as ristocetin, actinoidin, and teicoplanin, which inhibit bacterial cell wall synthesis by specifically binding to the acyl-D-alanyl-D-alanine carboxyl terminus of the muramyl-pentapeptide [2]. The binding of vancomycin and ristocetin to this cell wall component was first demonstrated by Reynolds [6]. Molecular models for the binding of peptides to vancomycin were later proposed by various groups [4,8]. Based on this mode of action, affinity chromatography was developed using D-alanyl-D-alanine as the ligand to selectively purify this class of antibiotics [3] as well as utilization of a tripeptide antagonism screen to identify glycopeptide antibiotics [5]. We report here the development of a peptide binding chromogenic assay useful in detecting and quantifying glycopeptides in fermentation broths.

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MATERIALS AND METHODS

Antibiotics and chemicals

Actinoidin, teicoplanin and avoparcin were kindly provided by the Institute of Antibiotics, Moscow, Gruppo Lepetit and Lederle Laboratories, respectively. Vancomycin, *N*-demethylvancomycin, actaplanin, A35512B, A47934 and A41030A were obtained from Lilly Research Laboratories. *Nα,Nε*-diacetyl-L-lysyl-D-alanyl-D-alanine was purchased from Bachem, Inc., Torrance, CA. Other antibiotics and synthetic peptides were commercially available from Sigma, St. Louis, MO.

Preparation of peptide and enzyme conjugates

L-Alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine was conjugated to bovine serum albumin at a 200:1 molar ratio (pentapeptide–BSA) and vancomycin to alkaline phosphatase at a 1:1 molar ratio via glutaraldehyde cross-linking [7].

Medium and antibacterial assay

Glycopeptide-producing cultures were grown in glucose-peptone broth (glucose 1%, black strap molasses 2%, Bacto peptone 0.5% and $CaCO_3$ 0.2%, pH 6.9) at 30°C, 250 rpm for 5 days. Antibacterial activity was detected in an agar disc diffusion assay using *Bacillus subtilis* ATCC 6633 [9].

Peptide binding assay

This is a solid-phase, heterogeneous binding assay in which the pentapeptide–BSA was immobilized onto the wells of a microtiter plate (1.34 μg protein/well) at 4°C for 18 h in $NaCO_3/NaHCO_3$ buffer, pH 9.6 [9]. The wells were washed four times with phosphate-buffered saline containing 0.01% Tween-20 (PBS-Tween). A blocking step using PBS-BSA (5%) for 1 h at 37°C helped to reduce nonspecific binding. Vancomycin–enzyme label was then added at 0.12 μg enzyme protein/well. The plate was incubated at 37°C for 2 h, washed four times in PBS-Tween, and reacted for 30 min with 0.3 ml of a 1 mg *p*-nitrophenyl phosphate/ml solution. The reaction was terminated by the addition of 0.05 ml 3 N NaOH, and total bound enzyme was spectrophotometrically determined at 410 nm using a microtiter plate reader.

RESULTS AND DISCUSSION

Glutaraldehyde, a dialdehyde, is a cross-linking agent commonly used to link proteins through their amino residues to form a Schiff's base [1]. In like manner, the free amino groups on BSA are randomly linked to the free amino moieties of the L-lysine and the L-alanine at the amino terminus of the pentapeptide. Vancomycin, similarly, is linked randomly through its free amino functions of the vancosamine and the glutamate residue in the nucleus to the amino groups of the alkaline phosphatase by glutaraldehyde.

A competitive assay was performed to detect the presence of glycopeptide antibiotics in a sample. One-tenth milliliter of a solution containing either antibiotic, synthetic peptide, or fermentation broth was added to wells previously coated with 0.2 ml of the pentapeptide–BSA. The glycopeptide competes with the vancomycin–enzyme label for the limited peptide binding sites on the well. The quantity of enzyme–label bound to the peptide decreased with increasing concentration of glycopeptide in the sample, resulting in reduced enzyme activity. Utilizing 80% B/B_0 (bound enzyme/maximum enzyme bound in the glycopeptide-free sample) as the cut-off for the lower detection limit of the assay, vancomycin and *N*-demethylvancomycin were detectable at 0.4 and 0.9 μg/ml, respectively (Fig. 1). The IC_{50},

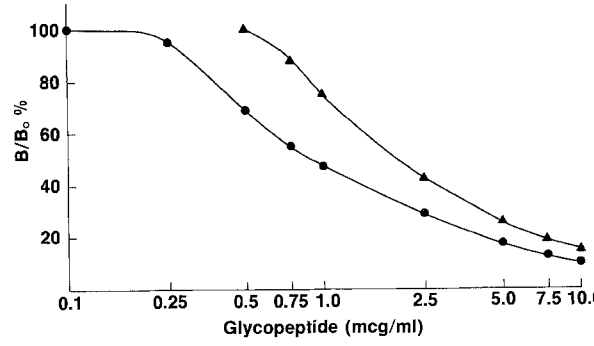

Fig. 1. Competitive peptide binding assay for vancomycin and *N*-demethylvancomycin. Utilizing 80% B/B_0 (bound conjugate/maximum binding) as the end point for the lower detection limit of this assay, vancomycin (●) and *N*-demethylvancomycin (▲) were detected at 0.4 and 0.9 μg/ml, respectively. IC_{50}, defined as the concentration of metabolite at 50% B/B_0, was 0.9 μg/ml for vancomycin and 2.0 μg/ml for its *N*-demethyl analog.

Table 1

Reactivity of antibiotics in the peptide binding assay

Antibiotic[a]	Zone of inhibition (mm)[b]	B/B_0[c] (%)
Benzylpenicillin	27	100
Tetracycline	23	100
Erythromycin	32	100
Gentamicin	20	100
Streptomycin	24	100
Novobiocin	11	100
Thiostrepton	trace	100
Monensin	10	100
Chloramphenicol	17	100
Bacitracin	–	100
Cycloserine	–	100
Polymyxin B	trace	100
Phosphonomycin	–	100
Actidione	–	100
Vancomycin	20	0.77
Control (PBS)	–	100

[a] Antibiotics tested at 100 μg/ml in PBS.
[b] Agar disc (6 mm) diffusion assay vs. *B. subtilis* ATCC 6633.
[c] B/B_0 = bound conjugate/maximum binding.

defined as the concentration of the metabolite at 50% B/B_0, generally reflects the affinity of the metabolite for the peptide. The IC_{50} values were 0.9 μg/ml for vancomycin and 2.0 μg/ml for its *N*-demethyl analog.

Antibiotics unrelated to glycopeptides, including cell-wall inhibitors such as benzylpenicillin, bacitracin, cycloserine, and phosphonomycin, did not bind to the peptide, suggesting that these antibiotics act via a different mechanism (Table 1). The ability of this assay to identify only vancomycin among the various non-glycopeptide antibiotics indicated that the assay is highly specific. The response of other members of the glycopeptide family was also examined in this assay. When tested in PBS solution, all glycopeptides reacted with the peptide in a dose-response fashion (Fig. 2). It appeared, however, that a different dose-response was observed with each glycopeptide. The assay sensitivity, which ranged from 0.25 μg/ml for actaplanin to 1.0 μg/ml for ristocetin, was comparable to that of the conventional antimicrobial bioassay. Since quantitative measurements of glycopeptides are feasible with this assay, we have provided an alternative method other than the commonly used agar diffusion assay for quantifying these antibiotics in fermentation or biological samples.

When known glycopeptide-producing cultures were grown in glucose-peptone broth, positive binding responses (B/B_0 < 80%) were observed in all fermentation samples, indicating the elaboration of glycopeptide antibiotics by these microorganisms (Table 2). Zones of inhibition exhibited by these producers were also included to indicate that antibacterial agents were produced. Since a negative response was observed with the uninoculated glucose-peptone broth, the complex organic constituents in

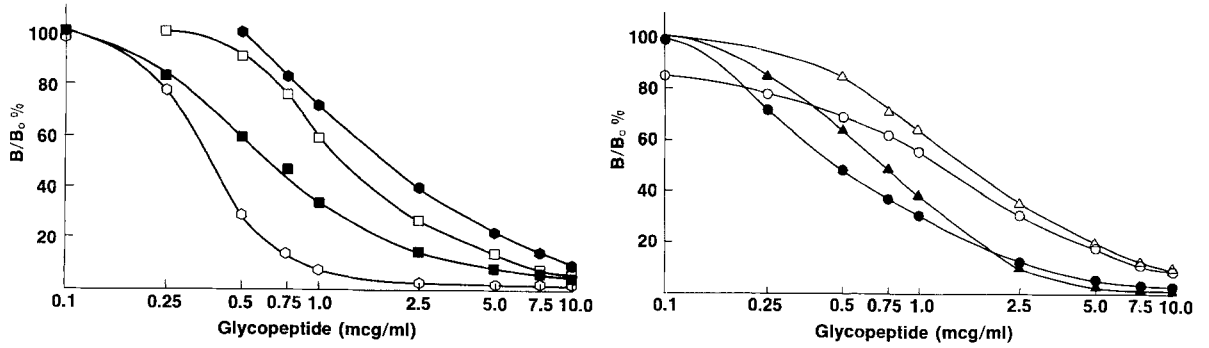

Fig. 2. Competitive peptide binding assay for glycopeptides. (○) actaplanin; (□) actinoidin; (■) avoparcin; (●) ristocetin A; (▲) teicoplanin; (⬢) A35512B; (△) A41030A; (○) A47934.

Table 2

Detection of glycopeptide producing cultures in fermentation. Cultures grown in glucose-peptide broth (250 rpm) for 5 days, at 30°C

Organism	NRRL	Glycopeptide	Zone of inhibition (mm)[a]	B/B_0 (%)[c]
Nocardia				
N. orientalis	2451	Vancomycin	30	4
N. orientalis	15232	*N*-Demethylvancomycin	26	5
N. sp.	8156	A35512	26	4
N. sp.	3218	Avoparcin	19	18
N. sp.	3582	LL-AM374	27	24
N. sp.	–	Ristocetin	16	16
N. lurida	2430	Ristocetin	trace	44
Actinoplanes				
A. missouriensis	23342[b]	Actaplanin	24	7
A. teichomyceticus	31121[b]	Teicoplanin	14	2
Streptomyces				
S. toyocaensis	15009	A47934	18	4
S. virginiae	15156	A41030	13	7
Control				
Glucose-peptone broth				100

[a] Agar disc (6 mm) diffusion assay vs. *B. subtilis* ATCC 6633.
[b] ATCC.
[c] B/B_0 = bound conjugate/maximum binding.

the broth did not interfere with this assay. It is, therefore, feasible to use this assay to detect glycopeptide-producing cultures in an antibiotic screening program and as a tool for strain improvement.

When synthetic peptides were tested in this assay instead of glycopeptides (Table 3), only those peptides containing a D-alanyl-D-alanine carboxyl terminus competed with the pentapeptide–BSA for the vancomycin–enzyme label, thus confirming that the D-alanyl-D-alanine portion of the peptide is required for the interaction. When *Micrococcus luteus* whole cells (2×10^7 cells/well) or a membrane preparation of this organism were immobilized on the wells instead of the pentapeptide–BSA, similar dose-response and assay kinetics were observed for vancomycin (data not included). However, 4 times the usual amount of vancomycin–enzyme label (0.48 μg enzyme protein/ml) was required in the assay. This may suggest that while vancomycin–alkaline phosphatase binds efficiently to the pentapeptide–BSA, it may be less readily accessible to the binding sites on the membrane or cell surface due to the size of this vancomycin–enzyme conjugate. However, free vancomycin bound readily to the bacterial cells as indicated in the peptide antagonism assay reported by Rake et al. [5]. This is an agar disc diffusion assay in which the antibacterial activity of glycopeptides was antagonized when bound to the structural analog of the peptide terminus of bacterial cell wall murein. This assay was reported to detect all known glycopeptides when present at 25 μg/ml. The peptide binding assay reported here operates on the same principle, except instead of detecting the antibacterial activities of these glycopeptides in the form of zone of inhibition, the binding of these metabolites to the cell wall analog was monitored. The sensitivity of this assay for most glycopeptides is below 1 μg/ml. This

Table 3

Reactivity of synthetic peptides in the peptide binding assay

Synthetic peptide[a]	μmol (μg/ml)	B/B_0[b] (%)
N-Acetylmuramyl-L-alanyl-D-isoglutamine (adjuvant peptide)	200	100
L-Alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine	200	8
	100	19
	20	66
	2	100
Nα,*Nε*-Diacetyl-L-lysine-D-alanyl-D-alanine	250	7
	125	17
	63	74
	25	100
D-Alanyl-D-alanyl-D-alanine	400	84
	200	100
Vancomycin	67 (100)	0.7
	33 (50)	1.5
	6.7 (10)	10.8
	0.67 (1)	41.4
	0.33 (0.5)	65.0
	0.17 (0.25)	91.6
	0.07 (0.1)	100
Control (PBS)	–	100

[a] Peptide tested in PBS.

[b] B/B_0 = bound conjugate/maximum binding.

glycopeptide assay provides a useful means for detecting and quantifying this class of metabolites in fermentation and biological samples.

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