IMPROVED SCREENING FOR β-LACTAM ANTIBIOTICS

A SENSITIVE, HIGH-THROUGHPUT ASSAY USING DD-CARBOXYPEPTIDASE AND A NOVEL CHROMOPHORE-LABELED SUBSTRATE†

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The very sensitive and specific method for the detection of β-lactam antibiotics using DD-carboxypeptidase (DDCase) from *Actinomadura* strain R39 has been improved to meet the requirements of a high-throughput β-lactam screening from culture broths of microorganisms. The method is based on a novel chromophor-labeled substrate *Nα*-acetyl-*Nε*-4-(7-nitrobenzofurazanyl)-L-lysyl-D-alanyl-D-alanine ($ANLA_2$) which is converted by DDCase into $ANLA_1$ with only one D-alanine residue left. Both compounds are intensely yellow as well as highly fluorescent and can be separated by thin-layer chromatography. This allows easy determination of residual DDCase activity after reaction with β-lactams by simple visual inspection of chromatograms. Also, many assays can be run at a time without sophisticated instrumentation. Details of the method as well as some results of a β-lactam screening performed with this type of assay are described.

Recently, FRÈRE *et al.*[1] described a rapid and sensitive assay for β-lactams which makes use of the ability of β-lactam antibiotics to inactivate the 53,000 dalton DD-carboxypeptidase (DDCase) from *Actinomadura* strain R39. This assay rests upon the determination of the residual enzyme activity after interaction with β-lactams using *Nα*,*Nε*-diacetyl-L-lysyl-D-alanyl-D-alanine as substrate. The amount of D-alanine liberated from Ac_2-L-Lys-D-Ala-D-Ala was determined by the D-amino acid oxidase/peroxidase/*o*-anisidine technique. It has been reported by FLEMING *et al.*[2], however, that this coupled enzyme assay is unsuitable for the detection of β-lactams in crude microbial broths because of adverse reactions of various broth constituents. In an effort to overcome this difficulty, the same authors successfully applied a coupled DDCase assay, using UDP-Mur-*N*-Ac-pentapeptide as a substrate. The conversion of this substrate by DDCase into the corresponding tetrapeptide was assayed by means of a HPLC technique. Since the isolation of UDP-Mur-*N*-Ac-pentapetide is rather laborious, we felt that the introduction of a suitable chromophore into synthetic α-acetyl-L-Lys-D-Ala-D-Ala might improve this target-directed assay. This report describes the successful application of this strategy for the synthesis of *Nα*-acetyl-*Nε*-4-(7-nitrobenzofurazanyl)-L-lysyl-D-alanyl-D-alanine ($ANLA_2$) as a novel substrate for DDCase, and the subsequent use of this substrate to establish a high-throughput screening for β-lactam antibiotics. Preliminary results of this screening are described.

† A portion of this work was presented at the 1983 Society of Industrial Microbiology Meeting in Sarasota, FL.

Materials and Methods

Reagents

$ANLA_2$ and $ANLA_1$ ($N\alpha$-acetyl-$N\varepsilon$-4-(7-nitrobenzofurazanyl)-L-lysyl-D-alanine) were prepared in our laboratories by conventional procedures. DDCase and diacetyl-L-Lys-D-Ala-D-Ala were obtained from UCB Bioproducts, Brussels, Belgium.

DDCase Assay

This assay was performed essentially as described in the legend to Fig. 2. Stock solutions of DDCase (1 mg/ml), and of $ANLA_2$ and $ANLA_1$ (both 2 mM) were made up in assay buffer (50 mM Tris-HCl, pH 8.3, containing 0.1 M NaCl and 5 mM $MgCl_2$[1]). When protected from light the substrate solution was completely stable at room temperature. The DDCase stock solution was divided into 50 μl portions and kept at $-20°C$ until used. The enzyme seems to be stable under these conditions for at least several weeks. Prior to use this stock solution was appropriately diluted 1/10 to 1/50 with buffer to achieve a linear reaction rate over 30 minutes at room temperature. The general procedures to assay β-lactam antibiotics with DDCase are given in the legend to Fig. 3. All assays were run in microtiter plates. For the quantification of $ANLA_2$ and $ANLA_1$ on TLC plates a Desaga model CD 50 densitometer with recording at 480 nm was used.

Results

The reaction scheme of the conversion of $ANLA_2$ to $ANLA_1$ by DDCase from *Actinomadura* strain R39 is shown in Fig. 1. Separation of the two compounds can be achieved by TLC. Since both compounds exhibit the same strong intrinsic fluorescence when exposed to UV light at 366 nm, they can easily be detected on chromatograms by simple visual inspection. In addition, $ANLA_2$ and $ANLA_1$ are intensely yellow-orange in color and can conveniently be quantitated by reflection densitometry. A representative densitometric scan is shown in Fig. 2. The time course of the DDCase-catalyzed hydrolysis of $ANLA_2$ was analyzed by drawing aliquots from the reaction mixture at appropriate time intervals. Substrate and product of the reaction were subsequently separated by TLC and the percentage of hydrolysis determined by densitometry. As can be seen from Fig. 3, the rate of hydrolysis was linear under these conditions for at least 30 minutes. Subsequent spraying of this plate to detect ninhydrin-positive compounds revealed that D-alanine was progressively released from $ANLA_2$ over the same period of time (data not shown). When the rate of DDCase-catalyzed release of D-alanine from $ANLA_2$ was compared with that from diacetyl-L-Lys-D-Ala-D-Ala as substrate, it was found that $ANLA_2$ was at least as good a substrate for DDCase as Ac_2-L-Lys-D-Ala-D-Ala (data not shown).

Fig. 1. Reaction scheme of the enzymatic conversion of $ANLA_2$ to $ANLA_1$ by DDCase.

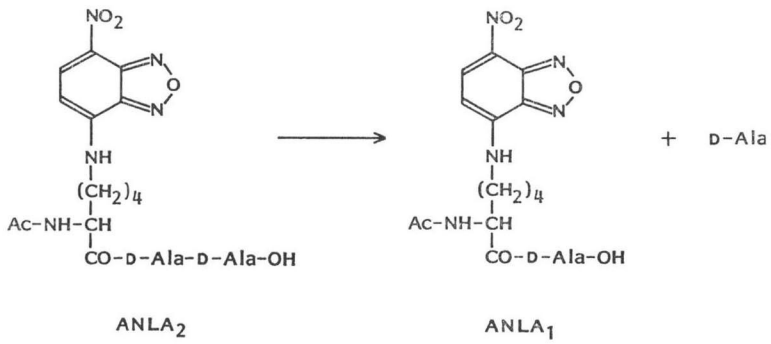


Fig. 2. Densitometric scan of the separation of $ANLA_2$ and $ANLA_1$.

The two compounds were applied onto silica gel plates (Kieselgel 60, Merck) and developed with the solvent system butanol - acetic acid - *n*-heptane (10: 8: 7). Scanning was performed with a Desaga model CD 50 chromatogram densitometer with absorption wavelength set at 480 nm.

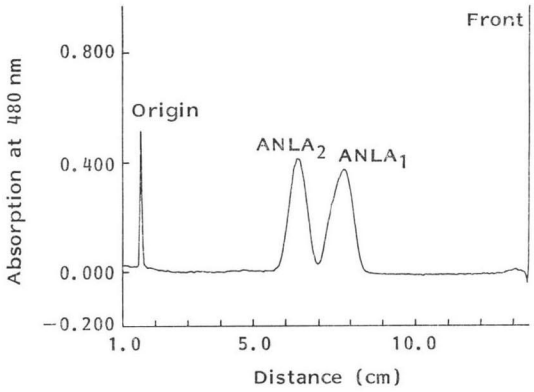
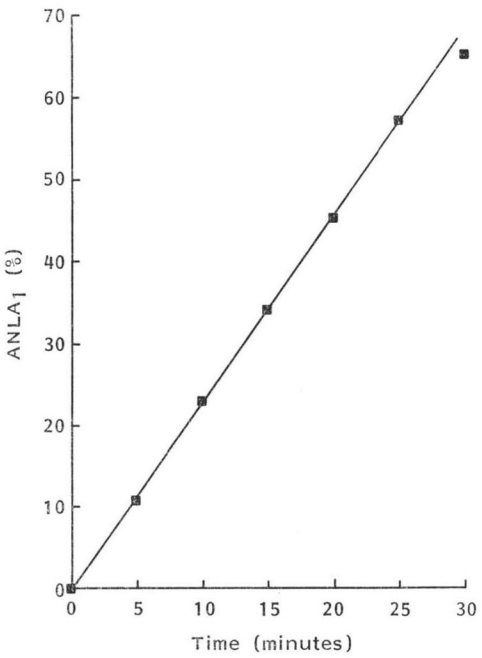


Fig. 3. Time course of DDCase-catalyzed $ANLA_2$-hydrolysis.

The reaction mixture (45 μl) consisted of: 50 mM Tris-HCl, pH 8.3, containing 0.1 M NaCl and 5 mM $MgCl_2$, 0.4 mM $ANLA_2$, and 14 μg of DDCase. The reaction was carried out at 37°C.

Samples (5 μl) were drawn at the indicated intervals and immediately applied onto silica gel plates (Kieselgel 60, Merck) to stop the reaction.

The plate was developed in solvent system butanol - acetic acid - *n*-heptane (10: 8: 7) and the percentage of $ANLA_1$ formed determined using the densitometric quantification as described in the legend to Fig. 2.



An example for the application of this assay to detect β-lactam antibiotics is shown in Fig. 4. Here, various concentrations of benzylpenicillin (10-fold geometric serial dilutions) were incubated with DDCase, and residual activity was subsequently determined using $ANLA_2$ as substrate. As is obvious from Fig. 4, no $ANLA_1$ is formed under these conditions as compared with the control reaction down to 0.5 μg of benzylpenicillin per ml of preincubation mixture, and gradual appearance of $ANLA_1$ is observed only at lower concentrations of benzylpenicillin. When a series of β-lactam antibiotics at various concentrations were tested under these conditions, the following I_{50} values (expressed as μg/ml of preincubation mixture for 50% inhibition) were determined by means of densitometry; benzylpenicillin (0.06), cephalosporin C (0.07), deacetylcephalosporin C (0.06), sulfazecin (0.11), 7-aminocephalosporanic acid (5.5), and nocardicin A (240). The non-β-lactam cell wall inhibitors vancomycin and flavomycin were not active in this assay.

Next, the DDCase/$ANLA_2$ assay was run in the presence of a variety of different unfermented media to check for possible interference with the assay, but virtually no unspecific effects were observed. On the other hand, when the broths of a series of standard β-lactam producing Streptomycetes including the carbapenem-producing strain Y-5633/mutant BL 36[3], and the sulfazecin producer *Pseudomonas acidophila* ATCC 31363[4] were investigated, a considerable DDCase-inactivating activity could always be detected even after a hundred-fold dilution of the broth. Notably, no interference by colored broth constituents was observed.

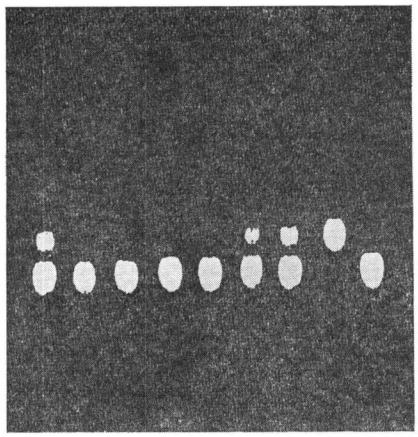
The subsequent screening of some 6,200 strains yielded 35 positive cultures. Interestingly enough,

Fig. 4. Residual DDCase activity after reaction with benzylpenicillin (10-fold geometric serial dilutions).

Ten microliters of freshly prepared solutions of benzylpenicillin in reaction buffer, or plain buffer for the control reaction, were mixed with 10 μl of DDCase (14 μg protein) and incubated for 10 minutes at room temperature. This preincubation mixture was supplemented with 25 μl of $ANLA_2$ stock solution and further incubated at 37°C for 20 minutes. The reaction was stopped by applying a 5 μl aliquot of the reaction mixture onto a silica gel plate (Kieselgel 60, Merck). The plate was developed in solvent system butanol - acetic acid - *n*-heptane (10: 8: 7).

Lane 1: control reaction without benzylpenicillin, Lane 2~7: 500, 50, 5, 0.5, 0.05 and 0.005 μg benzylpenicillin per ml of preincubation mixture, respectively. Lane 8: $ANLA_1$, Lane 9: $ANLA_2$.

The plate is viewed under UV light at 366 nm for better reproduction.



the incidence of positive cultures was relatively high with Streptomycetes and fungi. A large number of eubacteria screened resulted only in one positive isolate, whereas no DDCase positive culture was found within the *Micromonospora* genus. The compounds identified were penicillin N, 7-methoxydeacetylcephalosporin C, cephamycin A, *N*-acetylthienamycin, epithienamycin D and sulfazecin. The identification of three additional active compounds is still in progress.

Discussion

Many methods have been described in the literature for the detection of β-lactam antibiotics in culture broths of microorganisms. These include the use of morphological change-inducing activity[5], lack of antimicrobial activity against *Mycoplasma* strains[6], β-lactam antibiotic supersensitive mutants[7,8], β-lactamases as discriminating probes in combination with indicator strains[9,10], induction of β-lactamase in suitable strains[11], and *in vitro* β-lactamase inhibitory or inactivating activity[10,12,13]. All these assays can be regarded as indirect screening techniques since they are not, or not exclusively based on the actual mode of action of β-lactams *i.e.* inhibition of the enzymatic activity of penicillin-binding proteins (PBP's). Obviously, such a target-directed screening for β-lactam antibiotics would be preferable over the above mentioned assays in order to detect all compounds that specifically interact with PBP's, irrespective of their ability to penetrate into bacterial cells. Radioactive *in vitro* assays for the DD-carboxypeptidase activity of PBP 5/6 have been described[14]. However, they do not seem to be readily applicable to a microbial screening program. The recent discovery by FRÈRE *et al.*[1] that a soluble DDCase activity from *Actinomadura* strain R39 is quite sensitive to β-lactam antibiotics has been exploited by FLEMING *et al.*[2] to successfully screen for β-lactams in microbial broths. In an effort to improve this method, advantage was taken from data published by GHUYSEN *et al.*[15] who investigated the substrate profile of the R39 enzyme. They found that amongst synthetic substrates $N\alpha$-acetyl-L-Lys-D-Ala-D-Ala was split with the highest efficiency (V_{max}/K_m=3,000). Even though efficiency for diacetyl-L-Lys-D-Ala-D-Ala was considerably lower (V_{max}/K_m=410), this indicated, however, that a free ε-amino group of lysine was to a certain extent dispensible for this tripeptide to be a substrate. Surprisingly enough, introduction of the fluorescent nitrobenzofurazanyl chromophore into the ε-position of lysine of α-acetyl-L-Lys-D-Ala-D-Ala even improved the relatively poor affinity of this substrate to DDCase since the rate of DDCase-catalyzed liberation of D-Ala from both $ANLA_2$ and Ac_2-L-Lys-D-Ala-D-Ala was apparently the same. $ANLA_2$ as DDCase substrate, however, offers unique advantages; both the substrate and the product of the enzyme reaction can readily be detected on chromatograms by simple visual inspection due to their bright greenish fluorescence under UV light without the need of a multiple coupled enzyme assay[1]. Also, the use of highly

sophisticated instrumentation such as HPLC to assay DDCase[2] is dispensible. Scanning of chromatograms is only needed for accurate determinations of enzyme activities, *e.g.* the determination of I_{50} values for pure compounds. Scanning, however, is not necessary in a screening situation since the clear-cut visible plus/minus effect of $ANLA_1$ formation in the absence or presence of β-lactam antibiotics is sufficient for the detection of these compounds in culture broths of microorganisms. Since up to 20 assays can be analyzed on a single TLC plate, this assay provides the basis for a powerful high-throughput screening. The results of this screening confirm previous results[2], that the DDCase assay picks up all β-lactam compounds with antibacterial activity, the only exception being nocardicin. The screen has proven very promising and has yielded three strains producing novel β-lactam compounds that are currently under investigation.

Acknowledgments

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