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High-performance liquid chromatographic separation of the three stereoisomers of diaminopimelic acid in hydrolysed bacterial cells

M. ZANOL* and L. GASTALDO
Merrell Dow Research Institute, Lepetit Research Centre, 21040 Gerenzano (VA) (Italy)

ABSTRACT

A high-performance liquid chromatographic method able to separate the three stereoisomers of diaminopimelic acid (DAP) was developed. It consists of the reaction of DAP with 1-fluoro-2,4-dinitrophenylalanine amide (FDAA), followed by chromatographic analysis of the diastereomers under reversed-phase conditions. With this method whole cell hydrolysates of different bacterial strains were analysed. The DAP derivatives can be resolved from one another and from complex mixtures of other amino acids. The precision of the derivatization and the day-to-day reproducibility were calculated and were in the range 0.5–2%. The lower limit of sensitivity is *ca.* 600 ng for a mixture of the three isomers, which is sufficient for the detection of DAP even starting from small amounts of biological material. The method was applied to the analysis of the whole cell hydrolysate of *Kineosporia aurantiaca*, an unusual strain in which L,L- and "*meso*"-isomers are both present.

INTRODUCTION

Actinomycetes, the most important producers of antibiotics, have been taxonomically separated into groups by utilizing morphological, physiological and chemical criteria [1,2]. The chemical composition of the cell wall has been found to be a particularly useful tool in actinomycete taxonomy [3]. The majority of actinomycete strains have, as a cell wall constituent, a peptidoglycan containing diaminopimelic acid (DAP). This amino acid can occur in three isomeric forms, D,D, L,L and D,L (*meso*). The L,L-isomer is present in the cell wall of strains belonging to the genus *Streptomyces*, the most important antibiotic-producing actinomycetes, while other strains may contain the *meso* form. The separation of two of the isomers, the D,L and the L,L forms, in hydrolysates of whole cells has been reported using paper chromatography [4], thin-layer chromatography (TLC) [5] and high-performance liquid chromatography (HPLC) [6]. The D,D-isomer is not readily separable from the *meso* form and is of unknown taxonomic significance. Of these methods, HPLC gives a resolution superior to that of TLC, which is, however, still widely employed for rapid DAP isomer detection.

In this paper we report an improved HPLC separation method. It consists of a reaction with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) as described

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elsewhere [7], with consequent formation of diastereomers. The reaction is easy to perform and separation occurs in the reversed-phase mode without the employment of columns with optically active sites; the three isomers of DAP are clearly resolved. We applied it to two standard strains and to identify the DAP isomers of a new actinomycete strain, *Kineosporia aurantiaca* [8], which was isolated in our laboratories in the course of an extensive screening programme aimed at discovering new antibiotics from non-*Streptomyces* strains.

EXPERIMENTAL

Chemicals

DAP was obtained from Sigma (St. Louis, MO, U.S.A.) as a mixture of the three isomers (L,L, D,D and *meso*). FDAA was purchased from Pierce (Rockford, IL, U.S.A.). All the solvents and salts were of analytical-reagent grade.

Bacterial strains

The actinomycete strains utilized in the study were *Streptomyces coelicolor* ATCC 19832, *Nocardia lurida* ATCC 14930 and *Kineosporia aurantiaca* ATCC 29727. *Streptomyces coelicolor* and *Nocardia lurida* were chosen as markers for L,L- and *meso*-DAP, respectively.

Growth conditions

Stock cultures of microorganism were maintained on oatmeal agar slants and used to inoculate 500-ml erlenmeyer flasks containing 100 ml of V6 medium having the following composition: ACAS meat extract, 0.5%; yeast hydrolysate, 0.5%; peptone, 0.5%; casein hydrolysate, 0.3%; dextrose, 2%; sodium chloride, 0.15%. Flasks were incubated at 28°C for 3 days on a rotary shaker at 200 rpm, then the mycelium was collected by centrifugation and thoroughly washed with distilled water. The washed mycelium was resuspended in ethanol, dried under an air flow at room temperature and stored in dark vials.

Preparation of the whole cell hydrolysate

The whole cell hydrolysate was obtained according to Becker *et al.* [4]: *ca.* 20 mg of dried mycelium were placed in small ampoules with 1 ml of 6 *M* hydrochloric acid. The sealed ampoules were kept at 100°C overnight and, after cooling, the contents were filtered through Whatman No. 1 paper and the filtrate was evaporated to dryness under vacuum. The residue was dissolved in 0.3 ml of distilled water and used for the chromatographic analysis.

HPLC apparatus

A Model 1090 liquid chromatograph (Hewlett-Packard) equipped with a diode-array detector (DAD) and a Model 79994A workstation was used.

The chromatographic conditions were as follows: column, Ultrasphere ODS, 5 μm (25 cm × 4.6 mm I.D.) (Beckman); eluent, (A) 0.05 *M* triethylamine phosphate (pH 3)–acetonitrile (95:5) and (B) acetonitrile; flow-rate, 1 ml/min; gradient, 0 min 20%, 15 min 30%, 30 min 80%, 32 min 80%, 33 min 20% B and 34 min stop; DAD, monitored at 325, 254, 220 and 400 nm; UV spectra, measured between 220 and 450 nm.

Standard preparation

The derivatization procedure employs FDAA according to Marfey [7]: to 5 μmol of DAP in 100 μl of water, 100 μl of a 1% acetone solution of FDAA and 40 μl of a 1 *M* sodium hydrogencarbonate solution were added. After 1 h at 40°C, 20 μl of 2 *M* hydrochloric acid were added and, after degassing to remove bubles of carbon dioxide, the solution was injected under the conditions described above.

Sample preparation

To 50 μl of the hydrolysed whole cell, a saturated solution of sodium carbonate was added to bring the pH to 8–9. After addition of 100 μl of a 1% acetone solution of FDAA, the sample was treated as the standard and injected.

RESULTS AND DISCUSSION

A typical chromatogram of the standard stereoisomer mixture of DAP, derivatized with FDAA, is shown in Fig. 1. The peaks with retention times between 19 and 22 min are due to the three D,D-, D,L (*meso*) and L,L-stereoisomers. As no isolated stereoisomers are commercially available, peak assignment was achieved by comparison with hydrolysed whole cell samples of *Nocardia lurida* and *Streptomyces lividans*, which are known to contain D,L- and L,L-DAP, respectively. Figs. 2 and 3 show the chromatograms obtained. The peak at 19 min in Fig. 2 corresponds to the D,L-DAP derivative and that at 22 min in Fig. 3 is due to the L,L-DAP isomer. The reproducibility (precision) of derivatization of the standard solution was determined in the following way: two aliquots (1 and 2) of 5 μmol of DAP were derivatized as described above and injected five times each. The same samples were reinjected five times after 24 h. The precision is expressed as the relative standard deviation (R.S.D.) of the groups of

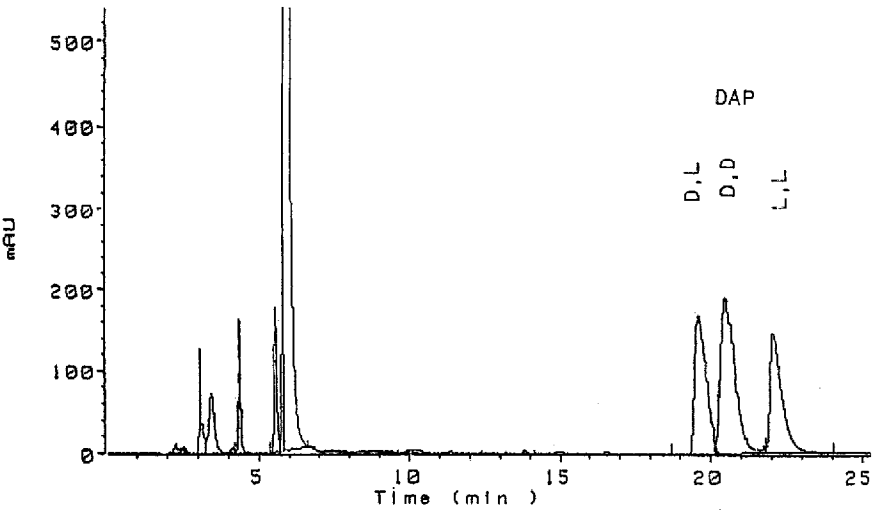

Fig. 1. Chromatogram of the three stereoisomers of diaminopimelic acid after derivatization with FDAA. An RP-18 column was used under gradient conditions. Detection at 325 nm.

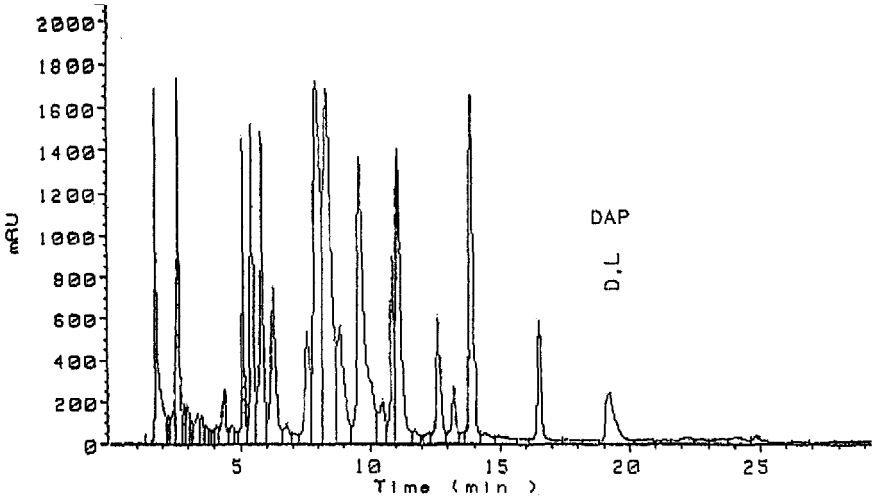

Fig. 2. Chromatogram of amino acid derivatives from whole cell hydrolysates of *Nocardia lurida*.

values reported in Table I. The instrumental precision, the reaction precision and the day-to-day reproducibility are therefore indicated.

The method was applied to verify the presence of DAP acid in *Kineosporia aurantiaca*. The hydrolysed whole cell clearly contained both the L,L and D,L forms. The resulting chromatogram is shown in Fig. 4.

The occurrence of a mixture of DAP isomers in the cell wall of bacteria is rare and in the actinomycetes is limited to the genus *Kitasatosporia* [9,10] in which the L,L form is present in major amounts in the aerial mycelium whereas the *meso* form is mostly present in the vegatative mycelium. *Kineosporia aurantiaca* does not develop

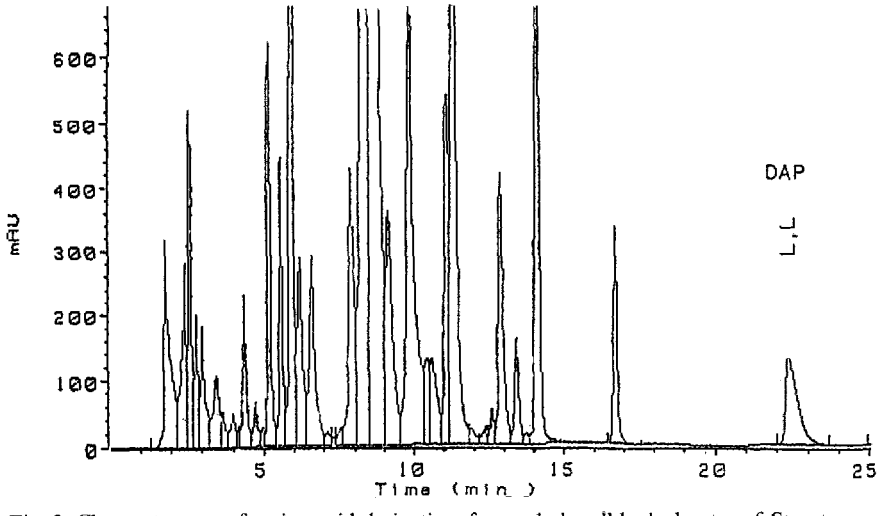

Fig. 3. Chromatogram of amino acid derivatives from whole cell hydrolysates of *Streptomyces lividans*.

TABLE I

PRECISION OF HPLC DETERMINATION OF DIAMINOPIMELIC ACID

Sample	Injections	R.S.D. (%)		
		D,L	D,L	L,L
1	5, 1st day	1.9	3.5	3.3
1	5, 2nd day	1.2	2.2	1.9
1	10, 1st + 2nd day	1.8	3.0	2,8
2	5, 1st day	0.4	1.0	1.0
2	5, 2nd day	1.1	2.6	2.7
2	10, 1st + 2nd day	1.3	1.9	1.5
1+2	10, 1st day	2.4	2.6	2.5
1+2	10, 2nd day	2.1	3.4	3.1

aerial mycelium but forms spores when grown on agar media. To check if the two isomers are present also during morphological cell differentiation, we separated by centrifugation and filtration the spores from the mycelium and both fractions were subjected to the hydrolysis. The result obtained with the spore-free mycelium fraction was the same as that obtained with the whole cell hydrolysate, indicating that both isomers are constitutively present in the cell wall. Attempts to identify DAP in purified spores failed even when a hydrolysate was obtained from *ca.* 200 mg of biomass (see Fig. 5). This indicates that the DAP spore content is at least two orders of magnitude lower than that of mycelium.

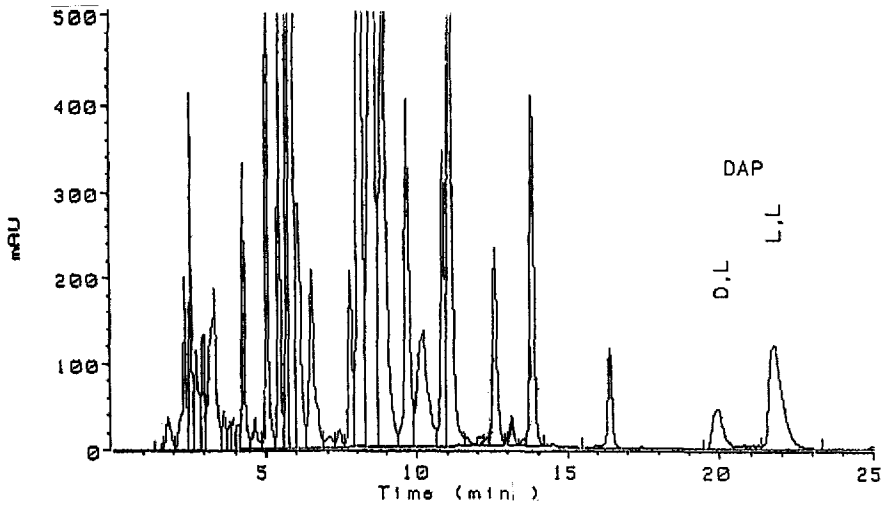

Fig. 4. Chromatogram of amino acid derivatives from whole cell hydrolysates of *Kineosporia aurantiaca*.

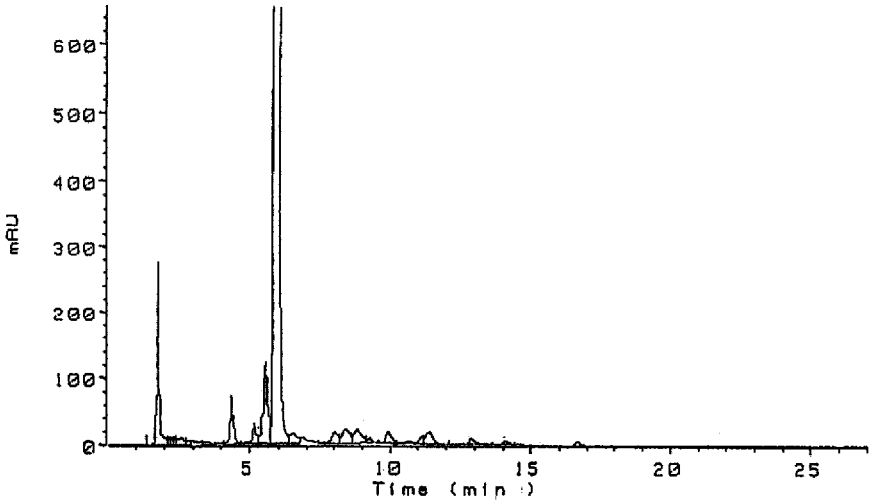

Fig. 5. Chromatogram of amino acid derivatives from purified spores of *Kineosporia aurantiaca*.

CONCLUSIONS

The method described here has several advantages: first, it is simple and reproducible, and second, very good resolution is obtained under the described chromatographic conditions. As can be seen in the figures, the peaks due to the three isomers of DAP are well resolved from one another and from other amino acids even in complex mixtures. Moreover, the derivatized diastereomers have a good response factor. The limit of detection is of the order of 600 ng of a mixture of the three isomers. The method is therefore sensitive enough to give information on DAP content starting from very small amounts of biological material. In addition, resolution of the D,D-isomer could provide further information of use in actinomycete taxonomy, given the hypothesis that this isomer may be present in some strains.

ACKNOWLEDGEMENTS

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