OPTICAL RESOLUTION OF 2,6-DIAMINOPIMELIC ACID STEREOISOMERS BY HIGH PERFORM- ANCE LIQUID CHROMATOGRAPHY FOR THE CHEMOTAXONOMY OF ACTI- NOMYCETE STRAINS

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For the chemotaxonomy of actinomycetes, we describe the optical resolution method for 2,6-diaminopimelic acid (DAP) stereoisomers by high performance liquid chromatography (HPLC) using 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC). The analysis was applicable both to whole-cell preparations and to cell wall ones. This method made it possible to analyze quantitatively LL-, DD-, and *meso*-DAP, compared with the previously reported methods of paper chromatography, thin-layer chromatography and HPLC.

2,6-Diaminopimelic acid (DAP) has three kinds of stereoisomers (LL-, DD-, and *meso*-DAP) and is contained in the cell-wall peptidoglycans of actinomycetes. General actinomycete strains contain LL- or *meso*-DAP. However, *Kitasatosporia* strains contain both LL- and *meso*-DAP (9). Also, some strains of the genus *Micromonospora* contain mostly *meso*-DAP and a small amount of both LL- and DD-DAP (5).

Rhuland described the method for resolving LL- and *meso*-DAP by paper chromatography (PC) (10). Cummins and Harris applied the analysis of DAP isomers in the cells to actinomycete taxonomy (3). After that, DAP isomers in the cells of actinomycete strains in various genera and species were studied by Yamaguchi (13), and Becker et al. (2). As a result, the determination of DAP isomers has been confirmed as an important criterion for the genus-level classification of actinomycetes (7).

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In general, PC and thin layer chromatography (TLC)(4) have been used to analyze DAP stereoisomers. These methods can separate LL-DAP from other DAPs, but not DD- from *meso*-DAP. Tisdall and Anhalt (12), and Iwami et al. (6) reported the method of resolving DAP stereoisomers by high performance liquid chromatography (HPLC) using dancyl chloride or *O*-phthalaldehyde. These analyses can be applied to the resolution of LL- and *meso*-DAP, but not to LL- and DD-DAP.

This paper deals with a new quantitative analysis of DAP stereoisomers for actinomycete chemotaxonomy by HPLC using 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC) which was developed by Nimura et al. (8).

MATERIALS AND METHODS

Reagents. Chemically synthetic DAP and GITC were obtained from Sigma Chemical Company (U.S.A.) and Chemi Sciences Industry Co. (Tokyo, Japan) respectively. DAP produced by a *Bacillus subtilis* lysine-requiring mutant was kindly supplied by Kyowa Hakko Kogyo Co. Ltd., Japan. All other chemicals were of reagent grade.

Equipment. The chromatographic system consisted of a high pressure pump equipped with a universal valve injector (Sanuki Industry Co., Tokyo, Japan), a Develosil ODS-5 column (100 $\times$ 6.0 mm I.D., particle size 5 μ m; Nomura Chemical, Seto, Japan) and an SPD-2A spectrophotometric detector (Shimadzu Corp., Kyoto, Japan).

Microorganisms. *Kitasatospora setae* KM-6054 (=ATCC 33774, =IFO 14216), *Streptomyces griseus* KA-1198 (ISP-5236), *Streptomyces albus* KA-1023 (ISP-5313) and *Nocardia asteroides* KB-87.

Sample preparation. Filamentous mycelia of *K. setae* were prepared as described previously (11). The whole cell and the cell wall of actinomycetes were prepared as described by Becker et al. (1) and Yamaguchi et al. (13), respectively. These hydrolysates were prepared according to the method of Becker et al. (2).

Derivatization. A chiral reagent, GITC, was used for derivatization. To 10 μ l of sample solution in 0.1 N HCl were added 20 μ l of 5% (v/v) triethylamine solution and 10 μ l of 2% (w/v) GITC solution in acetonitrile. The resulting mixture was allowed to stand at room temperature for 25 min. After 20 μ l of 50 mM taurine aqueous solution was added to the reaction mixture, it was allowed to stand at room temperature for 25 min. Five to 10 μ l of the resulting mixture was directly injected into an HPLC column.

RESULTS AND DISCUSSION

Figure 1 shows the separation of GITC derivatives of DAP stereoisomers by HPLC using a mixture of methanol and 20 mM ammonium phosphate (pH 5.0) (40:60) as a mobile phase. Chemically synthetic DAP and the DAP produced by *B.*

Fig. 1.

Fig. 2.

Fig. 1. Chromatogram of GITC derivatives of 2,6-diaminopimelic acid (DAP) stereoisomers.

(A), chemically synthesized DAP; (B), DAP produced by *Bacillus subtilis* (Lys⁻).

Mobile phase, methanol: 20 mM ammonium phosphate (pH 5.0)=40:60; flow-rate, 1.0 ml/min; detection, UV absorption at 250 nm; temperature, 45°C. See the text for peaks a-e.

Fig. 2. Chromatogram of the GITC derivatives of amino compounds in the hydrolysate of *Kitasatosporia* cell wall fraction. Sample, purified cell wall fraction from the filamentous mycelia of *K. setae* KM-6054.

Mobile phase, methanol: 20 mM ammonium phosphate (pH 5.0)=40:60; flow-rate, 1.0 ml/min; detection, UV absorption at 250 nm; temperature, 45°C.

subtilis (lys⁻) were used as the samples in Fig. 1(A) and (B) respectively. The highest peak b in Fig. 1(A) were assigned to *meso*-DAP, because chemically synthetic DAP consists of a mixture of LL-, *meso*- and DD-DAPs (1:2:1). Since *B. subtilis* (Lys⁻) is known to produce *meso*- and LL-DAP, the peak d in Fig. 1(B) was assigned to LL-DAP. As a result, the peaks of chromatogram in Fig. 1 were assigned as follows: the peaks a and d, LL-DAP; peaks b and e, *meso*-DAP; peak c, DD-DAP.

LL- and *meso*-DAPs in the hydrolysates of the *Kitasatosporia* cell-wall fraction were clearly separated as shown in Fig. 2. It was confirmed that the filamentous mycelia of *K. setae* KM-6054 contained mainly *meso*-DAP and a small amount of LL-DAP, as previously reported (11).

However, the DAPs in the hydrolysates of actinomycete whole cells, which are generally used for chemotaxonomy of actinomycete strains, cannot be separated by the HPLC described in Fig. 2. We examined the retention times of many kinds of amino acids under this condition. The retention times of DAPs were similar to L-

Fig. 3. Effect of the pH of the mobile phase on the separation of the GITC derivatives of the 2,6-diaminopimelic acid (DAP) stereoisomers, L-leucine and L-phenylalanine.

Flow rate, 1.8 ml/min; detection, UV absorption at 250 nm; temperature, 45°C.

Fig. 4. Chromatogram of the GITC derivatives of the standard 2,6-diaminopimelic acid (DAP) stereoisomer, L-leucine and L-phenylalanine.

Mobile phase, methanol: 50 mM acetate buffer (pH 3.2) = 40 : 60; flow rate, 1.8 ml/min; detection, UV absorption at 250 nm; temperature, 45°C.

leucine and L-phenylalanine. Figure 3 shows the effect of mobile-phase pH on the separation of DAP stereoisomers. Compared with the pH 5.0 system, the pH 3.2 system clearly separates the GITC derivatives of the DAP stereoisomers.

As shown in Fig. 4, the complete separation of the peaks of three kinds of DAP stereoisomers, L-leucine and L-phenylalanine, occurred under the condition of a

Fig. 5. Chromatogram of the GITC derivatives of the amino compounds in the hydrolysates of actinomycete whole cells. (A), *Kitasatospora setae* KM-6054; (B), *Streptomyces albus* KA-1023; (C), *Nocardia asteroides* KB-87.

Mobile phase, methanol: 50 mM acetate buffer (pH 3.0)=40:60; flow rate, 1.8 ml/min; detection, UV absorption at 250 nm; temperature, 45°C.

Table 1. Analysis of DAP isomers in whole-cell hydrolysates by HPLC and TLC.

	LL-DAP		meso-DAP		DD-DAP	
	HPLC (%)	TLC	HPLC (%)	TLC	HPLC (%)	TLC
<i>Kitasatospora setae</i> KM-6054	47	+	53	*	0	*
<i>Streptomyces albus</i> KA-1023	91	+	8	—	1	—
<i>Streptomyces griseus</i> KA-1198	92	+	6	—	2	—
<i>Nocardia asteroides</i> KB-87	0	—	96	*	4	*

* DAP spot was detected, but not identified.

+, detected; —, not detected.

mobile phase at pH 3.2.

Next the hydrolysates of actinomycete whole cells were analyzed by HPLC (Fig. 5). *K. setae* KM-6054 contains both LL- and meso-DAPs, *S. albus* KA-1023 contains mainly LL-DAP and *N. asteroides* KB-87 contains mainly meso-DAP. These were identical with the results of the TLC or PC analyses described previously. This confirms that this method is a useful way to analyze DAP stereoisomers for the chemotaxonomy of actinomycetes.

Since the standard curves for the GITC derivatives of meso-, LL-, and DD-DAPs by the HPLC analysis described above are linear, this method is useful for the quantitative analysis of the three kinds of DAP stereoisomers. Table 1 shows the quantitative analyses of three kinds of DAP stereoisomers in whole-cell hydrolysates by HPLC and the analysis by HPLC is compared with TLC. *K. setae*

KM-6054 contained 47% LL-DAP and 53% *meso*-DAP. The two *Streptomyces* strains used in this study contained small amounts of *meso*- and DD-DAPs which were not detected by TLC. *N. asteroides* KB-87 contained mainly *meso*-DAP and a small amount of DD-DAP. Since amino acids are racemized at a low rate in an acid hydrolysis process, it may be that these minor DAP isomers were produced by racemization. The precise origin of the minor DAP isomers are under investigation.

A new method of quantitative differential analyses for DAP stereoisomers by HPLC using GITC was established. Since the DAP stereoisomers in the hydrolysates of whole cells as well as the cell wall of actinomycetes can be analyzed, this method is useful for the chemotaxonomy of actinomycetes. Compared with TLC and PC which have been generally applied for chemotaxonomy, it is noteworthy that three kinds of DAP stereoisomers can be quantitatively measured in a short time by this method.

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