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RESOLUTION OF ALIPHATIC 2,4-DINITROPHENYL-DL-AMINO ACIDS ON A NATIVE CELLULOSE COLUMN*

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SUMMARY

Aliphatic DL-amino acids derivatized with fluorodinitrobenzene (2,4-dinitrophenyl-DL-amino acids) were resolved on a native cellulose (B-27) column. High resolution factors (α) and resolution rates (R_s) were obtained. It is suggested that an increase in the molecular size and an amplification of distortion resulting from the modification of the amino group may play an important role on their complete resolutions.

INTRODUCTION

Our primary concern in this work is to examine the critical mechanism for the resolution of DL-amino acids in a chiral environment. For the purpose of this study, a simplified system without any complicated modification can be recommended. We have already succeeded in resolving non-derivatized DL-amino acids by using native cellulose thin-layer and column chromatography[1–5]. Those previous reports gave evidence that the resolving power depends on the bulkiness of DL-amino acids[4] and the hydrophobicity of the environment[5]. It was also indicated that all the tested aliphatic DL-amino acids were not satisfactorily resolved because of their small side-groups[4].

We have therefore modified the amino group attached to the α-carbon atom with fluorodinitrobenzene to yield more bulky 2,4-dinitrophenyl (DNP)-amino acids. Such a modification should increase the asymmetry of the α-carbon atom, so that the modified racemic amino acids should be recognized more readily by cellulose molecules than the unmodified.

To date, there have been no reports published of the resolution of DNP-DL-amino acids. We describe here the resolution of aliphatic DNP-DL-amino acids by cellulose column chromatography.

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MATERIALS AND METHODS

Cellulose column

Specially manufactured native cellulose (tentative name: B-27, Japanese patent pending) was kindly provided by Daicel, Osaka, Japan. Another native cellulose (Lot No. 2331, Merck, F.R.G.) was also used in order to compare the resolution capability.

The cellulose was suspended in 1 *N* hydrochloric acid to eliminate some contaminants, and then washed with glass distilled water to remove the acid. The cellulose was then suspended in *n*-butanol containing ethanol (4:1, v/v) and packed into a glass column (60 × 0.85 cm I.D.; bed height, 50 cm). A shorter column (15 × 0.85 cm I.D.; bed height, 10 cm) was also used. The packed cellulose was washed with the same mixture of alcohols (4:1, v/v) in order to eliminate all traces of water, since the absolute amount of water in a column is the limiting factor for resolution.

Reactivation of cellulose column

Repeated use of the cellulose column leads to a decrease in the resolution capability, but it can be reactivated by the following elution procedure: (1) 5 ml of *n*-butanol; (2) 2 ml of 1 *N* hydrochloric acid; (3) 20 ml of water (approximately equivalent to a bed volume); (4) 10 ml of 0.1 *N* sodium hydroxide to neutralize residual hydrochloric acid; (5) 20 ml of water; (6) 5 ml of *n*-butanol; (7) 20 ml of *n*-butanol–ethanol (4:1, v/v) and (8) equilibration with 100 ml of the elution mixture, *n*-butanol–ethanol–water (4:1:0.1, v/v/v). When the column has been reactivated, perfectly resolved patterns are observed on chromatograms, so once the column has been packed it can be used repeatedly.

DNP-amino acids

Amino acids were purchased from Sigma (U.S.A.) and Wako (Osaka, Japan). A 6-mg amount of each DL-amino acid was dissolved in 1 ml of pure water, and 1 ml of 4 mg/ml of sodium bicarbonate was added. To this solution, 1 ml of 1-fluoro-2,4-dinitrobenzene ($5 \cdot 10^{-2}$ *M*) and 3 ml of ethanol were added. After mixing, the solution was warmed in a water-bath (80°C). In order to eliminate by-products that interfere with the chromatograms 35 min after the start, an aliquot (1 ml) of the reaction mixture was taken into a test-tube and completely dried at 60°C under nitrogen. Then 1 ml of 0.5 *N* hydrochloric acid was added to the tube and DNP-DL-amino acid was extracted with 4 ml of ether. The top quarter of ether phase was taken out and dried under nitrogen. This procedure was repeated twice. The dried sample was dissolved into 0.1 ml of 4 mg/ml of sodium bicarbonate, to which 5 ml of *n*-butanol was added. The sample solution thus prepared was used for the present experiment. However, the samples, *e.g.* alanine and α-aminobutyric acid, were not treated in this way.

Column chromatography

The cellulose column prepared by the above method was used for the present experiment. Prior to loading the sample solution, 5 ml of *n*-butanol were passed through the column to eliminate traces of water. Then, 0.05–0.1 ml of a sample solution containing 25–50 μg of DNP-DL-amino acids was eluted with the elution

mixture, *n*-butanol–ethanol–water (4:1:0.1, v/v/v). The eluent (flow-rate, 90 ml/h) was continuously monitored at 360 nm for all chromatograms.

Assignment of resolution of DNP-DL-amino acids

The resolved enantiomers were assigned by co-chromatography of different ratios of DNP-D- and -L-amino acids. For instance, 10 μg of DNP-L- and 30 μg of DNP-D-amino acids were coeluted. For the DNP-DL-amino acids tested, the L-enantiomers were eluted faster than the D-enantiomers. Circular dichroism (CD) measurements of the eluents can also be used for assignment provided a sufficient amount of the resolved enantiomers (more than 50 μg/ml) in available. The CD absorbance (θ value) was no greater than we expected, so we did not use CD measurements in the present study.

RESULTS AND DISCUSSION

Resolution of aliphatic DNP-DL-amino acids

Fig. 1 shows chromatograms of aliphatic DNP-DL-amino acids. The fact that the L-enantiomers were eluted faster than the D-enantiomers in co-chromatography

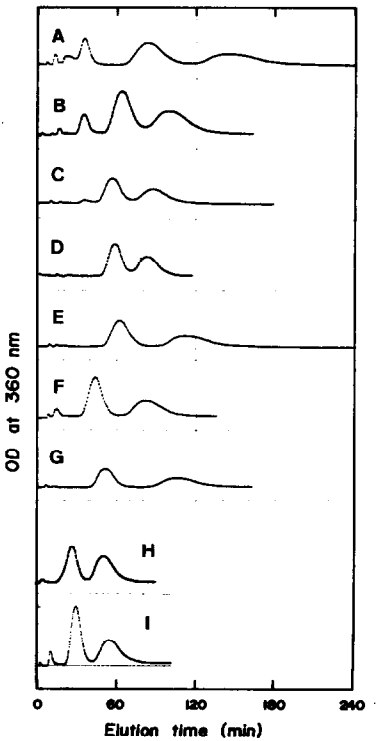

Fig. 1. Chromatograms of aliphatic DNP-DL-amino acids. (A) Ala (alanine); (B) AABA (α-amino-*n*-butyric acid); (C)nVal (normal valine); (D) nLeu (normal leucine); (E) Val (valine); (F) Leu (leucine); (G) Ile (isoleucine); (H) Ile; (I) Leu. Column, 50 × 0.85 cm I.D. (A–H) and 10 × 0.85 cm I.D. (I); applied sample, 25–50 μg; eluent, *n*-butanol–ethanol–water (4:1:0.1, v/v/v); flow-rate, 90 ml/h (A–G), 120 ml/h (H), and 30 ml/h (I). monitored at 360 nm (Hitachi Effluent Monitor, Tokyo, Japan); chart speed, 3 cm/h.

(data not shown) indicates that the latter interact more strongly with the cellulose. DNP-amino acids were not extracted with ether from the acidified sample solution (*cf.* Methods) peak(s) (30–40 min) apparently containing dinitrophenol and dinitroaniline was observed (A and B), and incomplete extraction also results in a small peak (C). However, no peaks of byproduct(s) appear when ether extraction is carried out (D–G).

By using different conditions from that for A–G, we obtained complete resolutions (H and I). Chromatogram I in particular has an extremely high theoretical plate number, because a shorter column was used.

Table I shows the resolution factors (α) and the resolution rates (R_s). Although non-proteinic amino acids gave relatively low values (α = 1.50, R_s = 0.97), others were satisfactory. The reason why we did not obtain high R_s values seems to be that the column was incompletely filled with cellulose; it is also possible that improved elutions conditions may lead to much higher values.

Content of water in the column and choice of elution mixture

In order to resolve DNP-DL-amino acids on the cellulose column, we had to pay particular attention to the content of water in both the column and the elution mixture, as the limiting factor. First of all, the washed cellulose powder should be dried overnight in an oven at 50°C to eliminate traces of water trapped in the cellulose. When a column is reactivated, extra care is necessary in washing out water from the cellulose. If the cellulose contains excess water, no resolution is observed. It should be mentioned that some non-aliphatic DNP-DL-amino acids (DNP-DL-Trp and -Phe, data not shown) can be resolved even with relatively high water content in the elution mixture, *n*-butanol–ethanol–water (4:1:0.25).

We used *n*-butanol–ethanol as the elution mixture, because water and/or etha-

TABLE I

RESOLUTION OF DNP-DL-AMINO ACIDS

$\alpha = (t_2 - t_0)/(t_1 - t_0)$, where t_0 = 7 min (void volume), and t_1 is for the peak of L-enantiomer and t_2 is for that of D-enantiomer. $R_s = 2\Delta t/(w_1 + w_2)$, where w_1 is for the theoretical base of the eluted L-enantiomer and w_2 is for that of D-enantiomer, and $\Delta t = t_2 - t_1$.

DNP-DL-amino acids	*Elution time (min)*		α	R_s
	t_1	t_2		
Ala	83	143	1.77	1.15
AABA	64	100	1.63	0.97
nVal	57	87.5	1.61	1.03
nLeu	58.5	83	1.50	1.11
Val	62.5	112	1.89	1.30
Leu	43	82	2.08	1.25
Ile	51	105	2.23	1.50
Ile*	26	50	2.26	1.16
Leu**	29	54	2.14	1.11

* Fig. 1H; t_0 = 6 min.
** Fig. 1I; t_0 = 7 min.

nol carries the sample to the void volume, but the sample cannot be eluted only with *n*-butanol. The elution mixture described here provides a relatively hydrophobic environment for cellulose, so that the samples were eluted sufficiently slowly.

Comparison of cellulose B-27 with another cellulose

We investigated another cellulose (Merck), but all the samples were eluted in the void volume and no resolved chromatograms were obtained. This is probably because we may not have completely eliminated the water from the cellulose. Cellulose B-27, on the other hand, seems to be easily dried by the present procedure. It can thus be concluded that the difference between the celluloses might be due to different water-retaining properties.

Evaluation

We have already resolved non-derivatized amino acids on native cellulose[4]. However, those resolutions took a long time (Leu and Ile, 10 h; nVal, nLeu and Val, 12 h; AABA, 15 h; Ala, 22 h) and required a long column (250 × 0.85 cm I.D.). Furthermore the resolutions were unsatisfactory, especially for alipohatic amino acids. We seem to have overcome these problems in the present studies. The results are quite reasonable, since amino acids become much more bulky and distorted after modification. The samples were quickly eluted, and the α-values and R_s values stand comparison with those obtained by high-performance liquid chromatography. Judging by its resolution capability and the modification process, the method reported here should be applicable to every laboratory study.

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