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Note

Direct liquid chromatographic resolution of (*R*)- and (*S*)-abscisic acid using a chiral ovomucoid column

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Abscisic acid (ABA) is a plant hormone with an important role in plant growth and development. It occurs naturally as the (+)-*S*-enantiomer. Most studies on ABA metabolism in plants have used racemic, synthetic material. As differences in the physiological effects and metabolism of enantiomers of ABA have been reported[1,2], the resolution of the enantiomers is essential for the elucidation of ABA metabolism in plants.

The enantiomers have only been separated with difficulty. Resolution has been achieved by repeated crystallization of brucine salts[3], acetylcellulose column chromatography[4], high-performance liquid chromatography (HPLC) of the methyl ester[5] and the diol derivative[6] and immunoaffinity chromatography[7]. Unfortunately, these methods are time consuming and unable to resolve ABA directly. Recently, Nonhebel[8] and Okamoto *et al.*[9] reported the direct optical resolution of ABA by HPLC using a chiral stationary phase, but the mechanisms involved in the resolution of (*RS*)-ABA have not been described. This paper describes the direct and rapid resolution of (−)-(*R*)- and (+)-(*S*)-ABA by HPLC using a chiral ovomucoid column[10,11]. The enantiomeric ratio can also be determined accurately by this method. Finally, the possible mechanisms involved in the resolution of (*RS*)-ABA are discussed.

EXPERIMENTAL

Apparatus

A Shimadzu LC-5A instrument equipped with an SPD-2A variable-wavelength UV monitor was used. A stainless-steel column (150 × 4.6 mm I.D.) was packed with ovomucoid-conjugated aminopropylsilica gel (5 μm), an available as ULTRON ES-OVM from Shinwakako (Kyoto, Japan).

Chemicals

Racemic ABA was purchased from Wako (Osaka, Japan). (+)-(*S*)-ABA was provided by Dr. N. Hirai. The methyl ester of (*RS*)-ABA was obtained by treatment of ABA with ethereal diazomethane. Sodium 1-heptanesulphonate (SHS) and tetra-*n*-

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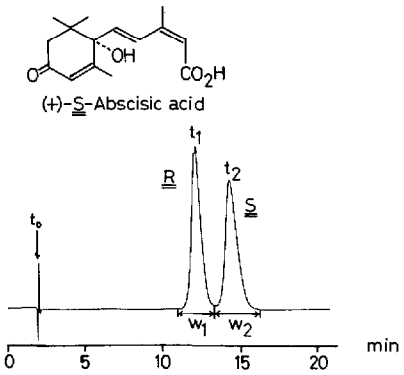

Fig. 1. Chromatogram resulting from an injection of 1 μl of a mixture of (*RS*)-ABA (0.5 μg). Mobile phase, 2% of 2-propanol in 20 m*M* potassium phosphate buffer (pH 3.50) at a flow-rate of 1.0 ml/min and ambient temperature. UV detection (254 nm, 0.04 a.u.f.s.). $k'_1 = (t_1 - t_0)/t_0$; $k'_2 = (t_2 - t_0)/t_0$; $\alpha = k'_2/k'_1$; $R_s = 2(t_2 - t_1)/(w_1 + w_2)$.

pentylammonium bromide (TPAB) were obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical-reagent grade.

RESULTS AND DISCUSSION

Fig. 1 shows the optical resolution of (−)-(*R*)- and (+)-(*S*)-ABA on an ovomucoid column. Baseline separation was almost achieved by using 2% 2-propanol in 20 m*M* potassium phosphate buffer (pH 3.50) as the mobile phase. The capacity factors (k') were 13.6 and 16.59, respectively. The separation factor (α) and resolution factor (R_s) were 1.22 and 1.01, respectively. The enantiomer with the longer retention time was identified as the natural *S*-enantiomer by co-chromatography with (+)-(*S*)-ABA standard.

We carried out tests on the recovery of the *R*-enantiomer from (*S*)-ABA to establish whether the enantiomeric ratio could be determined accurately. (*RS*)-ABA was added to (*S*)-ABA to give a concentration of (*R*)-ABA of 0.5 and 1.0%. The added *R*-enantiomer was recovered quantitatively at both concentrations by this procedure (Table I). The detection limit of (*R*)-ABA was 2.5 ng per injection. Hence the method allows the proportion of *R*-enantiomer in a sample to be measured precisely.

TABLE I

RESULTS OF TESTS ON THE RECOVERY OF (−)-(*R*)-ABA FROM (+)-(*S*)-ABA

Calculated (%)	*Found (%)*	*Recovery (%)*
0.99	1.00	101.0
	1.01	102.0
	Av. 1.01	Av. 101.5
0.50	0.50	100.0

TABLE II

INFLUENCE OF MONOVALENT ALCOHOLS AND ACETONITRILE ON THE RESOLUTION OF ABA

Mobile phase: modifier + 20 m*M* phosphate buffer (pH 3.50).

Modifier	*k'*	α	R_s
None	38.21	1.21	0.51
Methanol (7%)	15.96	1.16	0.61
Ethanol (5%)	10.61	1.00	–
2-Propanol (2%)	13.6	1.22	1.01
Acetonitrile (3%)	13.82	1.20	0.70

The retention and resolution of ABA can be regulated in three ways, either by addition of an organic modifier to the mobile phase, or by varying the ionic strength or the pH of the mobile phase. The content and kind of organic modifier in the mobile phase greatly influenced the retention and selectivity. An increased concentration of the organic uncharged modifier reduces the capacity factor. Table II gives some results obtained using monovalent alcohols and acetonitrile as an uncharged modifier. Ethanol removed the chiral resolution effect of the column, but the reason for this phenomenon is not clear. 2-Propanol was found to be the best modifier for ABA. ABA was strongly retained by the ovomucoid column at pH 4.0–5.0, as shown in Fig. 2. This pH range coincides with the isoelectric point of ovomucoid.

The above results imply that hydrophobic interactions are involved in the retention of the solute. Table II shows that the separation factors were almost unchanged despite about a three-fold reduction in the capacity factor on addition of 2% of 2-propanol. This suggests that hydrophobic interactions play an important role in the retention of ABA, but that they are not essential for the resolution.

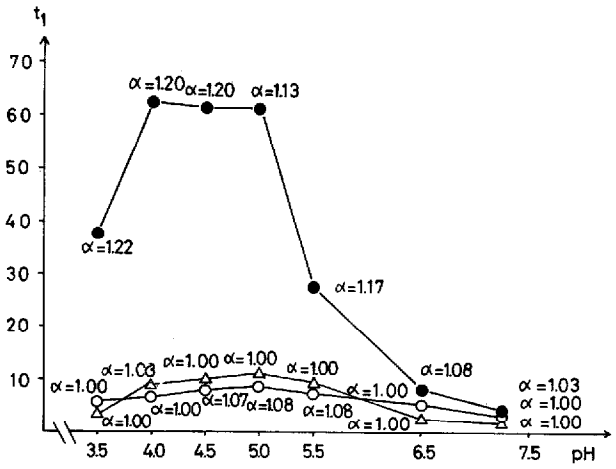

Fig. 2. Influence of pH and the charged modifiers, SHS and TPAB, on retention times (t_1) and separation factors (α). Mobile phase, potassium phosphate. ● = No modifier; ○ = 5 m*M* SHS added; △ = 5 m*M* TPAB added.

In a system with this chiral stationary phase, the addition of both SHS and TPAB charged modifiers to the mobile phase markedly reduced the capacity and separation factors (Fig. 2), as reported by Miwa *et al.*[11]. When SHS is used as anionic ion-pairing agent, the explanation for this phenomenon is as follows. 1-Heptanesulphonate ions may compete with ABA for the cationic cavity of the protein, so that the retention and resolution of ABA become small. With TPAB, the reason for the above observation is not yet clear. In both instances we could not improve the resolution by the use of additives, unlike the results obtained with Enantiopac[12].

The retention was also influenced by the ionic strength of the mobile phase. ABA exhibited a strong retention on this column at lower ionic strength, whereas the separation factors were almost unchanged (Fig. 3). This indicates that coulombic interactions, *i.e.*, an ion-exchange process, are involved in the retention of ABA, but that it is not so important for the chiral recognition and hydrophobic interaction with 2-propanol.

Variation of the pH of the mobile phase between 3.5 and 7.3 influences the capacity and separation factors of ABA, as demonstrated in Fig. 2. The separation factor decreases slightly with increasing pH, whereas the capacity factors are greatly affected. This effect seems to be caused by a change in the properties of the stationary phase with pH. As shown in Fig. 2, the enantiomers of ABA are best resolved at pH 3.50. Ovomucoid has an isoelectric point of 4.1 and has a net negative charge at lower pH. ABA is a weak acid having a pK_a of 4.8[13]. At pH 3.50 the carboxyl group in ABA appears to be almost undissociated. Hence strong hydrogen bonding between the carboxyl group in ABA and ovomucoid may play an important role in the chiral recognition. This also supports the fact that the methyl ester of (*RS*)-ABA shows a very poor resolution (R_s = 0.19) under the conditions used in Fig. 1.

In conclusion, fairly high separation factors can be obtained for the enantiomers of ABA by using ovomucoid as the chiral bonded phase. The optical purity of small amounts of ABA can be also determined directly, rapidly and accurately by this HPLC

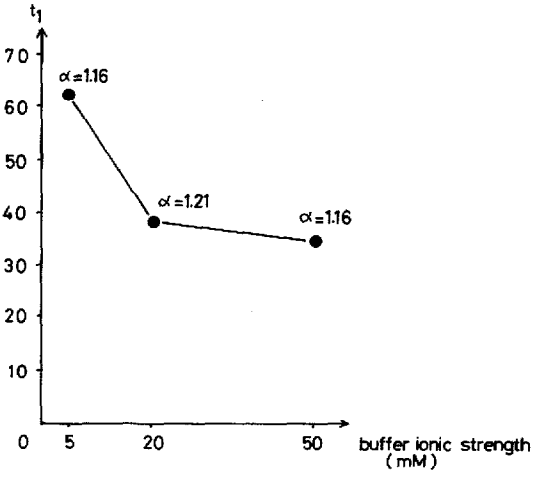

Fig. 3. Influence of buffer ionic strength (mM) on retention times (t_1) and separation factors (α). Mobile phase, potassium phosphate.

method. The experiments reported here indicate that strong hydrogen bonding may play an important role in the chiral recognition of ABA. Studies on the resolution of some ABA derivatives are in progress, and will be reported elsewhere.

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REFERENCES

1 N. Hirai, in N. Takahashi (Editor), *Chemistry of Plant Hormones*, CRC Press, Boca Raton, FL, 1986, p. 201.
2 J. A. D. Zeevaart and R. A. Creelman, *Annu. Rev. Plant Mol. Biol.*, 39 (1988) 439.
3 J. W. Cornforth, W. Draber, B. V. Milborrow and G. Ryback, *Chem. Commun.*, 3 (1967) 114.
4 E. Sondheimer, E. C. Galson, Y. P. Chang and D. C. Walton, *Science (Washington, D.C.)*, 174 (1971) 829.
5 I. D. Railton, *J. Chromatogr.*, 402 (1987) 371.
6 G. T. Vaughan and B. V. Milborrow, *J. Exp. Bot.*, 35 (1984) 110.
7 R. Mertens, M. Stuning and E. W. Weiler, *Naturwissenschaften*, 69 (1982) 595.
8 H. M. Nonhebel, *J. Chromatogr.*, 402 (1987) 374.
9 Y. Okamoto, R. Aburatani and K. Hatada, *J. Chromatogr.*, 448 (1988) 454.
10 T. Miwa, M. Ichikawa, M. Tsuno, T. Hattori, T. Miyakawa, M. Kawano and Y. Miyake, *Chem. Pharm. Bull.*, 35 (1987) 682.
11 T. Miwa, T. Miyakawa and M. Kawano, *J. Chromatogr.*, 408 (1987) 316.
12 J. Hermansson and G. Schill, in M. Zief and L. T. Crane (Editors), *Chromatographic Chiral Separations*, Marcel Dekker, New York, 1988, p. 245.
13 S. J. Neill and R. H. Horgan, in L. Rivier and A. Crozier (Editors), *Principles and Practice of Plant Hormone Analysis*, Vol. 1, Academic Press, London, 1987, p. 111.