Separation of Tryptophan from an Unidentified Compound (X 1) Produced from Indolepyruvic Acid by High-performance Liquid Chromatography

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Abstract Indole-3-pyruvic acid (IPA) is an unstable compound and some unidentified compounds (X 1~X 4) were naturally produced from IPA immediately after dissolving in 25% ethanol or after incubation in MB9 buffer solution at 39°C. Unfortunately, one of the unidentified compounds (X 1) overlapped with the peak of tryptophan (Trp), when analyzed by the previously reported method for analysis of Trp and its related compounds by high-performance liquid chromatography (HPLC). In the present study, a rapid method for the separation of Trp from X 1 was established using HPLC. LiChrospher 100 RP-18 column and methanol-50 mM sodium acetate buffer (pH 4.2) (20 : 80, v/v) as a mobile phase for isocratic elution were used for the analysis. The compounds were monitored at 280 nm with a UV absorbance detector. When a 5 μ l portion of deproteinized and filtered mixed sample of Trp and IPA was injected onto the HPLC system, the peaks of Trp and X 1 were separated within 7 min. The recovery of Trp was more than 96% and the minimum detectable limit was 40 pmol.

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Indole-3-pyruvic acid (IPA) is one of the precursors of tryptophan (Trp) biosynthesis. It is transaminated to form Trp by an enzyme, tryptophan transaminase (EC 2.6.1.27), where glutamate is amino group donor to IPA and it is in itself converted to 2-oxoglutarate. In Trp biosynthesis from IAA by ruminal bacteria, IAA has been observed to be reductively carboxylated to form IPA and then trans-

aminated to form Trp¹⁻³⁾. We have found that IPA was an unstable compound and it was naturally and rapidly converted to some unidentified compounds (X 1~X 4, unpublished data) even after dissolving in 25% ethanol and after incubation in MB9 buffer solution at 39°C, which has been used for incubation of rumen protozoa⁶⁾. As far as we know, there have been no reports on HPLC methods for the

インドールビルビン酸から生成される未同定化合物 (X 1) とトリプトファンの高速液体クロマトグラフィーによる分離 : ナジムディン モハマド・小野寺良次・溝上宗尚・カーン ロキブル イスラム (宮崎大学農学部, 宮崎市 899-2192)

determination of IPA.

Recently an HPLC method for the analysis of Trp and its ten related compounds has been established⁵⁾. With this method⁵⁾, however, the peaks of Trp and one (X1) of the unidentified compounds produced from IPA, when dissolved, have empirically known to overlap each other. Therefore, it is essential to develop a method for the separation of Trp from X1 in order to study Trp biosynthesis from IPA. In the present paper, a newly established method for the separation of Trp from X1 and the simultaneous quantitative determination of Trp by HPLC was studied.

Materials and Methods

Apparatus

A liquid chromatography pump (CCPM-II), variable wavelength ultraviolet detector (UV-8020), column oven (CO-8020), degasser (SD-8022) and autosampler (AS-8020) were obtained from Tosoh (Tokyo, Japan); the data analyser (Chromatopac, C-R 3A) was from Shimadzu (Kyoto, Japan) and the LiChrospher 100 RP-18 column (250×4 mm I.D.) of 5 μ m particle size was from Kanto Chemical (Tokyo, Japan) under the licence of E. Merck (Darmstadt, Germany).

Chemicals

L-Tryptophan was purchased from Wako Pure Chemical Industries (Osaka, Japan); IPA from Sigma (St. Louis, Mo, USA) and sulfosalicylic acid (SSA) from Nacalai Tesque (Kyoto, Japan). Ultra pure water made with Milli-Q Labo (Nihon Millipore Kogyo, Tokyo, Japan) was used to prepare the mobile phases and other solutions.

Sample preparation

Indole-3-pyruvic acid was dissolved in 25% ethanol. One-ml portion of 20 mM IPA solution was added in 19 ml MB9 buffer solution⁶⁾ and incubated it at 39°C for 14 h in 30 ml Ehrlenmeyer flask. Samples were collected after 1, 2, 3, 4, 5, 6, 7 and 14 h interval, mixed with an equal volume of 4% (w/v) SSA and

filtered through a 0.45 μ m membrane filter (Toyo Roshi Kaisha, Tokyo, Japan) before HPLC operation. Portions (5 μ l) of the filtrate were directly injected onto the HPLC system.

Chromatography

The mobile phase used for isocratic elution was methanol : 50 mM sodium acetate buffer (pH 4.2) (20 : 80, v/v) and the pH was adjusted to 4.2 by 50 mM acetic acid. Before use, the mobile phase was filtered with a membrane filter (HV 0.45 μ m, Nihon Millipore Kogyo, Tokyo, Japan). The flow rate was 1.0 ml/min; column temperature, 40°C; monitoring wavelength at 280 nm with a UV absorbance detector; column, LiChrospher 100 RP-18 (250×4 mm I.D.) of 5 μ m particle size.

Results and Discussion

In the present method, interference from compounds other than UV-absorbing substances such as aromatics could be easily avoided by the choice of a suitable monitoring wavelength (280 nm). The methanol concentration and pH value of the mobile phase influenced the retention time of the peaks of Trp and the unidentified compound (X1) produced from IPA. In the present experiment, the methanol content and pH of the mobile phase were changed from 60 to 0% and from 3.6 to 5.6, respectively. As a result, when methanol : 50 mM sodium acetate buffer (pH 4.2) (20 : 80, v/v) was used, the peaks of Trp and the X1 were efficiently separated within 7 min (Fig. 1). This method can also separate Trp from its related 10 compounds reported by Mohammed *et al.*⁵⁾, but the disadvantages of this method are that the retention time and minimum detectable limit were longer and lower, respectively, than those of the previously reported method⁵⁾. With the increasing methanol percentage and pH of the mobile phase, retention times of Trp and X1 shortened and overlapped each other.

A linear relationship was obtained between the peak-heights and the different

Fig. 1. Chromatogram of Trp (1 mM standard solution) mixed with equal volume of 1 mM IPA after 2 h incubation in MB9 buffer solution at 39°C and then deproteinized.

Mobile phase : methanol : 50 mM sodium acetate buffer (pH 4.2) (20 : 80, v/v).

Abbreviations : SSA=sulfosalicylic acid, IPA= indolepyruvic acid, Trp=tryptophan, X1=unidentified peak 1, X2=unidentified peak 2, X3=unidentified peak 3, X4=unidentified peak 4.

concentrations of standard compound (Trp), and thus the concentration of Trp in the sample which contains X1 and other related compounds can easily be calculated from their peak height. The minimum detectable limit of Trp was 40 pmol and the correlation coefficient between peak height and concentration was 0.99999.

The peak purity was confirmed by comparing the peak-height ratio of Trp, added in IPA solution at different wavelengths (270/280 nm) according to the report of Lefeng *et al.*⁽⁴⁾. Different concentrations of Trp were added in IPA solution and the analytical recovery was calculated by comparing the concentration of Trp found in the mixture (Trp and IPA) with

Fig. 2. Conversion of IPA in MB9 buffer solution to unidentified compounds (X1~X4) during incubation at 39°C for 14 h.

X1=unidentified peak 1, X2=unidentified peak 2, X3=unidentified peak 3, X4=unidentified peak 4.

the standard concentration added in it. The recovery of Trp of the sample containing X1 was more than 96%. Precision is expressed as the coefficient of variation and the accuracy as the bias. The coefficient of variation and bias range for four different concentrations of Trp added in IPA solution were varied between 0.19 to 3.35% and 0.13 to 3.84%, respectively. The observed concentrations were in good agreement with the actual concentrations.

This method was applied to investigate the tendency of conversion of IPA to some unidentified compounds in MB9 buffer solution after incubation at 39°C for 14 h. When 1 mM of IPA was incubated in MB9 buffer solution at 39°C for 14 h, four unknown peaks X1, X2, X3 and X4 appeared, and peaks of X3 and X4 seemed to overlap with unknown compounds. Before incubation, only a small peak of X1 was found on the chromatogram and after 1 h incubation, small peaks of X3 and X4 appeared. A small peak of X2 appeared after 2 h incubation, but then it decreased gradually. The highest peak of X1 was found after 2 h incubation and then

the peaks of X1 and X2 gradually decreased, and X3 and X4 gradually increased with the incubation time (Fig. 2).

We have tried to identify the four unknown peaks. Among them, the peak of X2 overlapped with the peak of indolelactic acid, X3 with indoleacetic acid and X4 with indolealdehyde. X1, however, could not be identified as IPA itself, because IPA seemed not to dissolve so quickly as to be detected as a peak in HPLC, even though the IPA solution in 99.5% ethanol was injected onto HPLC immediately after dissolved. It is assumed that X1 and X2 are gradually converted to X3 and X4 with incubation time, though we could not identify each compound.

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References

- 1) Allison MJ, Robinson IM. Tryptophan biosynthesis from indole-3-acetic acid by anaerobic bacteria in rumen. *Biochem. J.*, 102 : 36-37. 1967.
- 2) Allison MJ, Robinson IM, Baetz AL. Tryptophan biosynthesis from indole-3-acetic acid by anaerobic bacteria from the rumen. *J. Bacteriol.*, 117 : 175-180. 1974.
- 3) Kristensen S. Ruminal biosynthesis of aromatic amino acids from arylacetic acids, glucose, shikimic acid and phenol. *Br. J. Nutr.*, 31 : 357-365. 1974.
- 4) Lefeng Z, Yunlu Y, Ruiyu Y. Direct determination of phenylalanine in serum extracts of phenylketonuria patients by reversed phase high-performance liquid chromatography. *J. Chromatogram.*, 282 : 333-339. 1983.
- 5) Mohammed N, Onodera R, Khan RI. A new determination method of tryptophan and its related compounds by high-performance liquid chromatography and its application to rumen fluid. *Anim. Sci. Technol. (Jpn.)*, 69 : 1020-1028. 1998.
- 6) Onodera R, Henderson C. Growth factors of bacterial origin for the culture of the rumen oligotrich protozoon, *Entodinium caudatum*. *J. Appl. Bacteriol.*, 48 : 125-134. 1980.