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Note

Liquid chromatographic resolution of hypoglycin A from leucine

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Hypoglycin A (HG-A) or 2-amino-4,5-methylenehex-5-enoic acid is the natural toxic compound found in ackee fruit. Because of the toxicity of HG-A, there is great concern about the existence and content levels of HG-A in canned ackee fruit if immature fruit is packed. Since current liquid chromatographic methods do not give baseline resolution of HG-A from leucine, methodology is needed to accurately resolve and measure HG-A in ackee fruit products.

Early work on HG-A quantitation relied upon paper chromatography. Patrick[1] tried 17 different paper chromatographic systems and failed to separate HG-A from leucine. Other paper chromatographic methods[2,3] which appear to work are lengthy and unreliable.

Published procedures utilizing ion-exchange amino acid analyzer (IE-AAA) systems are not adequate for HG-A quantitation. Scott *et al.*[4] developed a method for the determination of HG-A using a Beckman IE-AAA system and post-column ninhydrin reaction. However, HG-A and leucine were not baseline resolved. Fincham[5] used an indirect AAA method to quantitate HG-A in ackee fruit with HG-A and leucine coeluting as one peak. The peak was measured before and after destroying the HG-A by bromination. The resulting peak was leucine with the difference between the nonbrominated and brominated samples being HG-A. The author indicated that the method is not precise because of the differential method of analysis[5]. Attempts to use the bromination procedure in our laboratory were unsuccessful. This paper describes an IE-AAA system which gives baseline resolution of HG-A from leucine and 16 other amino acids.

EXPERIMENTAL

Reagents

Buffers. The IE-AAA buffers were pre-prepared (Pickering Labs., Mountain View, CA, U.S.A.): (1) buffer A-Na 315 (0.2 *M* sodium citrate, pH 3.15); (2) buffer B-Na 740 (0.9 *M* sodium chloride–sodium acetate, pH 7.40).

Ninhydrin. Trione ninhydrin reagent (Pickering Labs).

HG-A standard solution. Dissolve HG-A in buffer A (72.6 μg/ml). Make further dilutions with buffer A to give 18.15, 12.10 and 4.84 μg/ml. Note: Hypoglycin A standard is not available commercially. The standard used for this study was provided by Dr. Tanaka, Yale University School of Medicine.

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Amino acid standard mixture. Pre-prepare a mixture of 17 amino acids and ammonia to give 0.5 μmol amino acid/ml (No. 20079, Pierce, Rockford, IL, U.S.A.).

Amino acid standard mixture/HG-A. Combine equal volumes of the HG-A standard and amino acid standard mixture (0.25 μmol amino acid/ml).

Method development standard mixtures. Make up a 0.5 μmol/ml solution of L-methionine, L-isoleucine, L-leucine and L-tyrosine (No. 185009, 185006, 185007, and 185015, respectively, Pierce) in buffer A. Combine equal volumes of this mixture with HG-A standard.

Apparatus

As amino acid analyzer a Waters Assoc. ion-exchange ninhydrin detection system was used. It consisted of a 25 cm × 4.6 mm amino acid column (Waters No. 80002), two Model 510 pumps, a temperature control module (TCM) system, a Model A-30-SW ninhydrin pump, a WISP Model 710B auto sample injection system, a Model 440 detector, and an interface module system. The system was operated by a Digital Pro 350 computer with a Digital LA50 printer.

Chromatographic conditions

Instrument parameters. Injection volume, 15 μl; flow-rate, 0.4 ml/min; detector wavelength, 436 and 546 nm (additive); column temperature, 62°C; reaction oven temperature, 120°C; ninhydrin flow-rate, 0.4 ml/min.

Mobile phase. Buffer A–buffer B (30:70), set pump A (buffer A) at 30% and pump B (buffer B) at 70% of the total flow-rate of 0.4 ml/min for baseline reolution of HG-A and leucine.

System calibration

Inject the high standard solution (72.6 μg/ml) to calibrate the system and establish baseline, peak response and retention time parameters. These parameters were entered into the computer program. Linearity and detection limits were established by running a series of four standards in duplicate ranging from 4.84 to 72.6 μg/ml.

RESULTS AND DISCUSSION

The binary buffer gradient systems recommended by the manufacturer for separation of 17 amino acids and ammonia in 110 min failed to resolve HG-A and leucine. A method development standard mixture which incorporated those amino acids immediately preceding and following HG-A elution (methione, isoleucine, leucine, and tyrosine) was used. Various modifications in the binary gradient failed to resolve HG-A and leucine; however, a small distortion in the HG-A/leucine peak was observed during an isocratic segment in one of the gradient program modifications, where the ratio of buffers A and B was 80:20. This small distortion of the HG-A/leucine peak was believed to be a crude initial separation between HG-A and leucine.

This was further investigated by trying different isocratic buffer systems. Fig. 1 shows the progressive improvement in the resolution of HG-A and leucine by using isocratic buffer systems consisting of 50:50, 40:60 and 30:70 mixtures of buffers A and

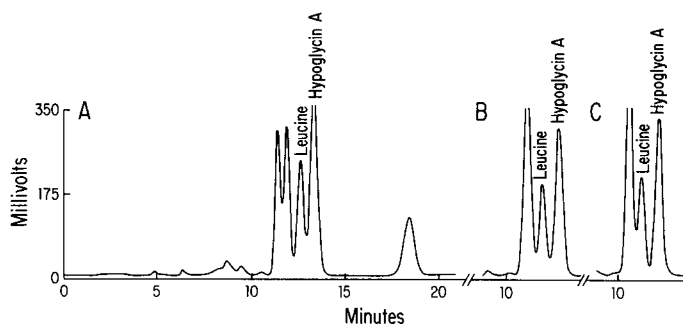

Fig. 1. Chromatogram of method development standard mixture of methionine, isoleucine, leucine, HG-A and tyrosine with a mobile phase of varying buffer A–buffer B ratios at a flow-rate of 0.4 ml/min. Elution order: Methionine, isoleucine, leucine, HG-A and tyrosine. (A) 50:50; (B) 40:60; (C) 30:70.

B. The 50:50 mixture of buffers A and B resolved all four amino acids of the method development standard with partial coelution of HG-A and leucine (Fig. 1A). As the amount of buffer A decreased, the methionine and isoleucine peaks coeluted and HG-A and leucine resolution increased (Fig. 1B). Optimal baseline resolution of HG-A and leucine was obtained with the isocratic buffer system made of a 30:70 mixture of buffers A and B (Fig. 1C). This buffer system was further evaluated using a standard mixture of the 17 naturally occurring amino acids. Fig. 2 is the chromatogram of the 17 amino acid mixture with HG-A. In a separate chromatogram not shown here, the 17 amino acid mixture without HG-A was run and showed that there were no coeluting amino acids with HG-A.

Evaluation of the method showed a linear peak response to increasing HG-A concentrations from 4.84 to 72.6 μg/ml (72.6–1089 ng/15 μl injection volume) with a correlation coefficient of 0.999. The precision of the system was evaluated by analyzing the high standard (72.6 μg/ml) five times and gave a peak area of 7.920 $\pm$ 0.0147 (0.19% C.V.) and indicated good reproducibility.

This method presents a rapid, reliable procedure for the baseline resolution of HG-A and leucine and will serve as a basis for the study of HG-A content in ackee fruit.

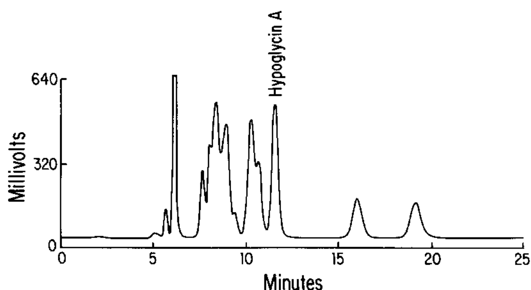

Fig. 2. Chromatogram of 17 naturally occurring amino acids with HG-A. Mobile phase, buffer A–buffer B (30:70) at a flow-rate of 0.4 ml/min.

REFERENCES

1 S. J. Patrick, *Can. J. Biochem. Physiol.*, 40 (1962) 1195.
2 D. E. Abrahams and E. A. Kean, *West Indian Med. J.*, 18 (1969) 147.
3 E. V. Ellington, *West Indian Med. J.*, 10 (1961) 184.
4 P. M. Scott, H. G. Botting, P. C. Kennedy and J. E. Knipfel, *J. Food Sci.*, 39 (1974) 1057.
5 A. G. Fincham, in E. A. Kean (Editor), *Pan-American Association of Biochemical Societies Symposium*, Vol. 3, *Hypoglycin*, Academic Press, New York, 1975, pp. 21–30.