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Note

Analysis of *o*-phthalaldehyde derivatives of acidic and polar amino acids in fermentation broths by high-performance liquid chromatography

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The high-performance liquid chromatographic (HPLC) separation of *o*-phthalaldehyde (OPA) derivatives has been used widely for the analysis of amino acids. In general, the OPA–HPLC method has been developed for the analysis of amino acids in either protein hydrolyzates[1–5] or physiological fluids[2–8], and it has served also as an assay of enzyme activities[9–11] and, to a more limited extent, has been applied to the analysis of amino acids in culture broths or cell extracts of microorganisms[3,12]. The latter two applications present different analytical problems from the first two in that quantitative determination of only one or two amino acids may be required instead of twenty or more. A substantial saving in both time and materials would be achieved if a more rapid OPA–HPLC method were available for less complex analyses. Such a method has been achieved in this investigation by using a short column and by varying the mobile phase composition to optimize the separation of groups of amino acids with similar polarities. This approach has been used routinely for the analysis of acidic and polar amino acids, particularly to monitor the production of 5-hydroxy-4-oxonorvaline (HON) by fermentation[13].

EXPERIMENTAL

Chemicals

OPA reagent containing mercaptoethanol (Fluo-R) was obtained from Beckman (Mississauga, Canada). HPLC-grade methanol and tetrahydrofuran (THF) were supplied by BDH (Toronto, Canada). ACS reagent-grade sodium acetate and sodium periodate were obtained from Anachemia (Montreal, Canada) and Fisher (Ottawa, Canada), respectively. Water was purified by means of a Nanopure system (Sybron/Barnstead, Boston, MA, U.S.A.). HON was prepared by fermentation[13] and all other amino acids were purchased from Sigma (St. Louis, MO, U.S.A.).

Chromatographic equipment

The Beckman HPLC system consisted of two Model 110B pumps, a Model 340 gradient mixer equipped with a Model 210A injection valve fitted with a 20-μl sample loop, and a Model 157 filter fluorometer equipped with a 9-μl flow cell and filters for excitation at 305–395 nm and emission at 420–650 nm. An ECS XT Model 5151 computer(Electronic Control Systems, Mississauga, Canada) with a 20-MB hard disk,

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was interfaced (Computer Interface Consultants, Armdale, Canada) to the pumps and detector for gradient control, data collection and data analysis.

Chromatographic conditions

Separations were obtained on a Beckman ODS Ultrasphere column (5 μm, 45 × 4.6 mm I.D.) connected to an Upchurch Model C-130B guard column (20 × 2 mm I.D.) packed with Perisorb RP-18 (30–40 μm, Chromatographic Sciences Co., Montreal, Canada). The total flow-rate was 2.5 ml/min and gradients were formed between two water-aspirator degassed solvents: 0.1 *M* sodium acetate (adjusted to pH 6.2 with 3 *M* HCl)–methanol–THF (900:95:5), and methanol.

Derivatization

Derivatization was performed by adding Fluo-R OPA reagent (40 μl) to a mixture of sample (20 μl) and cysteic acid internal standard (20 μl, typically 50 nmol/ml) solutions. After 1 min at ambient temperature the reaction was quenched with 0.1 *M* sodium acetate buffer (120 μl, pH 6.2) and a portion (20 μl) was chromatographed.

Internal standard correction

An average response (peak area/nmol) for the internal standard, cysteic acid, was calculated from the cysteic acid peak areas of twenty injections at three different concentrations (48, 24 and 10 nmol/ml). The ratio of the cysteic acid peak area in sample chromatograms to the average cysteic acid response was used to correct other peaks for the variability of the derivatization reaction. The average relative standard deviation for the peak area of cysteic acid was 3% (15 injections).

Calibration of fluorescent response by periodate oxidation

A 90-h culture of *Streptomyces akiyoshiensis*[13] was centrifuged (15 850 *g*) and a stock solution of HON was prepared by dilution of 5.0 ml of the supernate to 50.0 ml with water. A portion of the stock solution was chromatographed to obtain the fluorescent response of HON. Aqueous sodium perodate (50 μl, 47 m*M*) was added to a second portion (500 μl) of the stock solution and, after 15 min at ambient temperature, the periodate reaction mixture was diluted with water and chromatographed to measure the fluorescent response of the aspartic acid derived from HON. The procedure was repeated with dilutions of the stock solution. A separate calibration curve prepared from L-aspartic acid was used to calculate the amount of aspartic acid obtained from HON and thus the original HON concentration.

RESULTS AND DISCUSSION

The OPA–HPLC method has been developed extensively for the separation of OPA derivatives of amino acids in complex mixtures (*e.g.*, protein hydrolyzates and physiological fluids) using 150- or 250-mm reversed-phase columns, but the analysis times associated with these procedures is long (>15 min)[1–10]. A way to shorten the analysis time was indicated by Jones and Gilligan[2,3] who demonstrated that the length of the column does not greatly affect the resolution of the OPA derivatives of amino acids and that higher flow-rates at lower column pressures were possible using a short

column. This approach has been developed in this investigation to provide a more rapid separation (5 min) of 17 of the 18 protein amino acids that form fluorescent derivatives with OPA using a shorter (45 mm) and less expensive column (Fig. 1a).

Although glutamine and histidine were not resolved and a baseline separation has not been achieved for all 18 protein amino acids, the procedure (Fig. 1a) is useful for the analysis of samples in which glutamine has been converted to glutamic acid, *e.g.*, protein hydrolyzates, and for rapid screening of complex mixtures. In addition, cysteine can be detected after oxidation to cysteic acid[14].

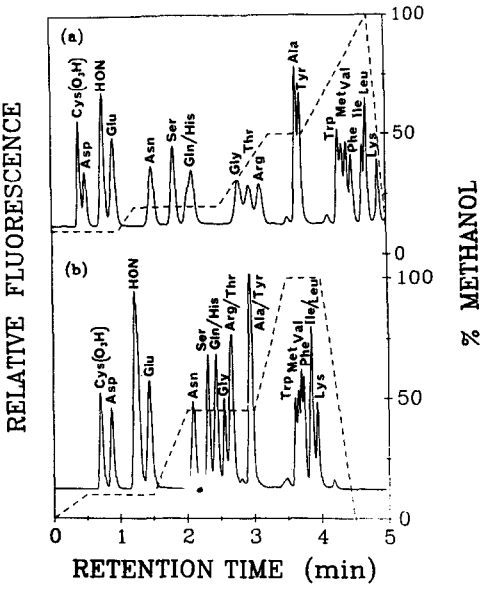

Fig. 1. Separations of a standard mixture of amino acids. (a) Optimized gradient (dashed line) for the separation of 18 amino acids. (b) Optimized gradient (dashed line) for the separation of acidic and polar amino acids.

For the analysis of a few components of a complex mixture or for the analysis of a less complex mixture (*e.g.*, fermentation broth), the gradient conditions shown in Fig. 1a can be modified to provide an improved separation of amino acids with similar polarities while retaining the 5-min run time. However, a loss of resolution of amino acids outside the selected group is observed. For example, an improved separation of tyrosine and alanine was observed when the second plateau of the gradient presented in Fig. 1a was increased from 20 to 25% methanol, but the separation of glycine, threonine and arginine was adversely affected.

To achieve a better separation for the analysis of non-polar amino acids (Trp, Met, Val, Phe, Ile and Leu) and lysine, a gradient that went from 0 to 50% methanol in the first minute, remained at 50% methanol for 2.5 min, and increased to 100% methanol over the next 1.5 min before returning to 0% methanol, was used.

The selective analysis of acidic and polar amino acids (Fig. 1b) was developed to

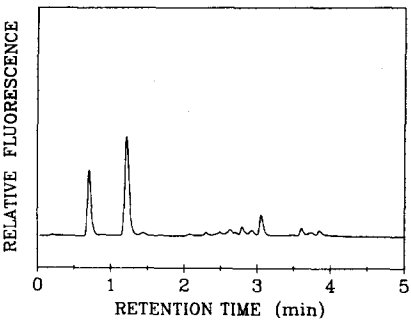

Fig. 2. Chromatogram of a 72-h culture broth showing HON (retention time 1.3 min, 8 m*M*) as the major amino acid and cysteic acid (retention time 0.8 min) as the internal standard. Gradient conditions are as shown in Fig. 1b.

monitor the production of HON by fermentation (Fig. 2). A pH of 6.2 was found to give the optimum separation of cysteic acid and aspartic acid as well as HON and glutamic acid. Since the retention times of aspartic acid and glutamic acid are sensitive to pH changes, they are incompletely resolved from cysteic acid and HON, respectively, at pH 6.5 and higher.

The gradient conditions presented in Fig. 1b permitted the rapid determination of the acidic and polar amino acids in less than 1.5 min. By increasing the methanol content of the mobile phase the less polar amino acid derivatives were eluted and the column was prepared for the next injection within 5 min and a waiting period was not required between injections. A disadvantage of the OPA precolumn method is the reagent peaks that appear on aging of the OPA reagent, but these have longer retention times (2.8, 3.8, 4.0 and 4.2 min) and do not interfere with the analysis of acidic and polar amino acids.

Fermentation broths containing HON were analyzed directly without any prior cleanup and without interferences (*e.g.*, Fig. 2) due to the selectivity of the OPA method for primary amines. The column lasted for hundreds of injections over a period of a year provided the guard column was changed regularly. Cysteic acid, an internal standard with a rapid retention time to minimize interference with other amino acids, was added to each sample to improve the reproducibility for quantitative determinations[2].

Since HON undergoes partial decomposition during isolation, an analytically pure sample was not available and an alternative method was used to calibrate the fluorescent response of the OPA derivative of HON. Mild oxidation of HON with periodate produced aspartic acid[13,15], a readily avalaible standard. HPLC monitoring of the periodate cleavage reaction showed that HON was completely and rapidly consumed and that aspartic acid was the only product observed. As shown in Fig. 3, the fluorescent response of the aspartic acid derived from HON by periodate (upper line) was directly related to the fluorescent response of HON (lower line); both are proportional to concentration. If undetected products were formed by side reactions or further oxidation, then these would have produced a more pronounced effect at the lower HON concentrations since a constant amount of periodate was used for each HON concentration. However, the linear responses in Fig. 3 for HON and aspartic acid derived from HON preclude the formation of other products in any significant

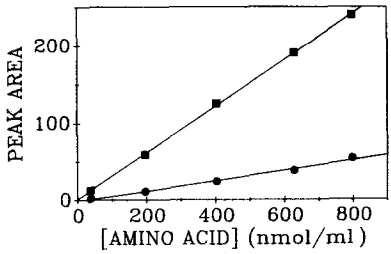

Fig. 3. Calibration curve for HON (●, $r = 0.9959$). Points (■) on the upper line ($r = 0.9998$) represent the amount of aspartic acid formed by periodate cleavage of HON and correspond to the points on the lower line. Each point is the average peak area obtained from three injections.

amounts. Moreover, there was no detectable change in the concentration of aspartic acid in the presence of periodate over a period of 50 min.

The different slopes of the aspartate and HON lines in Fig. 3 (aspartate–HON ratio $= 4.5 \pm 0.6$) demonstrate that a greater fluorescent response is obtained for aspartic acid and that, in general, calibration of the fluorescent response for each individual amino acid is necessary for accurate quantitative results. The lower fluorescent response associated with HON could be due to a lower yield of the OPA derivative, to a rapid decomposition of the OPA derivative[16], to non-optimized excitation and emission wavelengths, or to the decomposition of HON during the derivatization reaction. The derivatization reaction is carried out at pH 9.5 and the base lability of γ-oxo amino acids has been described[17].

The procedure described has proved to be very useful for the analysis of acidic and polar amino acids in culture broths[13], for monitoring the isolation of amino acids[13], and for the measurement of enzyme activity in crude cell extracts[11]. Also, this selective and rapid method may be useful for amino acid analysis in other biological fluids such as cerebrospinal fluid[8] and for the analysis of strongly acidic amino acids[18].

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