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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ε-(γ-GLUTAMYL)LYSINE AND MONO- AND BIS-γ-GLUTAMYL DERIVATIVES OF PUTRESCINE AND SPERMIDINE

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SUMMARY

A sensitive, simple, and rapid high-performance liquid chromatographic (HPLC) method is reported for the determination of ε-(γ-glutamyl)lysine and certain γ-glutamylpolyamines in selected fractions from ion-exchange chromatograms of protein digests. The method involves pre-column derivatization of the γ-glutamylamine conjugates with *o*-phthalaldehyde, linear-gradient reversed-phase HPLC separation, and fluorimetric detection. The gradient used was designed to provide a means of avoiding a desalting step, while maintaining proper chromatographic performance. γ-Glutamylamines in amounts from 0.1 to 1 nmol display linear concentration–response relationships. The detection limits are approximately 10 and 200 pmol per mg of protein for the γ-glutamylpolyamines and for ε-(γ-glutamyl)lysine, respectively. The use of the method is exemplified by an analysis of the epidermal cell envelope from the skin of a newborn mouse.

INTRODUCTION

γ-Glutamylamines occur as components of proteins both in cells and extracellularly. It is well established that transglutaminases catalyze formation of the covalent γ-glutamyl amide bond by means of a calcium-dependent acyl transfer reaction, in which the γ-carboxamide group of peptide-bound glutamine residues are the acyl donors and the primary amino groups in a variety of compounds serve as acyl acceptors[1,2]. Among the γ-glutamylamines found in mammalian proteins are ε-(γ-glutamyl)lysine[3], mono- and bis(γ-glutamyl)polyamines[4–7], and γ-glutamylhistamine[8]. Of these, ε-(γ-glutamyl)lysine and the bis(γ-glutamyl)polyamines functions as covalent crosslinks between protein chains.

Methods for the determination of the γ-glutamylamine content of proteins rely upon a means of liberating the intact γ-glutamylamines from the protein chains. Exhaustive digestion by proteolytic enzymes has proven satisfactory for this purpose,

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since γ-glutamyl bonds are resistant to proteolysis[3,4]. Several methods have been reported for the measurement of ε-(γ-glutamyl)lysine[3]. The ion-exchange chromatographic method can be sensitive when measurements are made by means of a fluorogenic reagent, such as *o*-phthalaldehyde (OPA). However, the method is limited by the lack of complete resolution of ε-(γ-glutamyl)lysine from certain amino acids, especially when the ratio of dipeptide to amino acids is low. Although this problem can be overcome by rechromatography, this discourages the use of the procedure for the analysis of the many samples needed for some investigations. High-performance liquid chromatographic (HPLC) procedures with precolumn derivatization by OPA promise to provide rapid, simple, and sensitive alternatives to the ion-exchange method, although reports of their application thus far have been limited.

An automated ion-exchange column-chromatographic method for γ-glutamylpolyamines proved useful for the assay of those compounds that were radiolabeled in the polyamine portion[4]. Although only the most basic of the γ-glutamylpolyamines were sufficiently separated from amino acids to allow their direct determination in unlabeled form, indirect assay was possible by measurement of polyamines liberated upon acid hydrolysis or by treatment with γ-glutamylamine cyclotransferase, an enzyme specific for the release of amines from γ-glutamylamines[9].

The intent of the present study was to develop a simple and sensitive HPLC method that permits the direct determination of both ε-(γ-glutamyl)lysine and the γ-glutamylpolyamines. The primary objective of this method is to provide a means of assessing the covalent crosslinks of those protein materials known to be, or believed to be, polymerized through the catalytic action of transglutaminases. A pattern of γ-glutamylamines in the epidermal cell envelope from skin of the newborn mouse is presented as an example of the applicability of the method.

EXPERIMENTAL

Materials

OPA was purchased from Dionex (Palo Alto, CA, U.S.A.), N-(γ-glutamyl) putrescine from Vega (Tucson, AZ, U.S.A.), amino acid standard H [2.5 μmol/ml of each amino acid, except cystine (1.25 μmol/ml)] from Pierce (Rockford, IL, U.S.A.), acetonitrile and water, HPLC grade, from Burdick & Jackson (Muskegon, MI, U.S.A.). N^1,N^4-bis(γ-glutamyl)putrescine[10], N^1- and N^8-(γ-glutamyl)spermidines[10], N^1N^8-bis(γ-glutamyl)spermidine[10], and ε-(γ-glutamyl)lysine[11] were synthesized by the referenced procedures.

Apparatus

Analyses were performed with a Waters HPLC system (Waters Assoc., Milford, MA, U.S.A.), consisting of a Model 6000A solvent delivery system, a Model U6K sample injector, a Model 660 solvent programmer, and a Model 420E fluorescence monitor.

Chromatographic conditions

Reversed-phase separations were accomplished at room temperature in a 30 cm × 3.9 mm I.D. μBondapak C_{18} 10-μm column (Waters Assoc.), using a linear gradient from 10 m*M* sodium acetate (10 m*M* acetic acid to pH 5.0 with 5 *M* sodium

hydroxide) to 10 mM sodium acetate–acetonitrile (20:80, v/v) over a period of 33 min at a flow-rate of 1.0 ml/min.

Instrument setting

The fluorescence detector excitation wavelength was set at 340 nm (12 nm bandpass) and the intrinsic fluorescence was monitored at 440 nm (>440 nm bandpass). The detector sensitivity was set at gain 16.

Standard solutions and calibration

Stock solutions of γ-glutamylamines were prepared with water at concentrations of *ca.* 0.01 *M* and frozen at −20°C. Precise concentrations were determined by measurement of amino acid or polyamine components following acid hydrolysis. Dilution of stock solutions with water were made prior to use. Standard curves were prepared, using γ-glutamylamine samples in the range 0.1–1.0 nmol and plotting peak height *versus* amount of sample.

Sample preparation

The methods for exhaustive proteolytic digestion of protein samples and for automated ion-exchange chromatography of the digests have been published[4,10]. Appropriate fractions from the ion-exchange column were combined and the pH of the combined fractions was adjusted to *ca.* 7 with 1 *M* sodium carbonate. Equal volumes of an OPA reagent [22.4 m*M* OPA and 0.4 *M* sodium carbonate in 50% (v/v) methanol containing 2% (v/v) ethanethiol, prepared fresh daily] were added to aliquots of these solutions. After incubation for 5 min at room temperature, portions of the solutions of OPA derivatives (5–500 μl) were injected directly into the HPLC system.

RESULTS AND DISCUSSION

HPLC separation

The ion-exchange column-chromatographic system[10], used preliminary to the HPLC analysis, provides excellent separation of most of the γ-glutamylamines under consideration and thus has been useful in the measurement of these compounds in protein digests when they are radiolabeled specifically in their amine portion. However, the ion-exchange system does not allow satisfactory separation of ε-(γ-glutamyl)lysine or certain of the γ-glutamylpolyamines from all amino acids, and therefore it is not directly applicable to determination of these compounds in digests of unlabeled protein material. The HPLC system, developed here in order to obtain the desired separation from amino acids, provides the means for direct identification and measurement of the γ-glutamylamines.

The HPLC elution pattern of the OPA derivatives of ε-(γ-glutamyl)lysine and various γ-glutamylpolyamines is shown in Fig. 1. The elution positions of the derivatives of lysine and of the unique amino acid hypusine[12] are also presented in Fig. 1 to exemplify the effectiveness of the HPLC system in defining several compounds that are not satisfactorily separated by the ion-exchange chromatograph. Efficient resolution of N-(γ-glutamyl)putrescine, N^1,N^8-bis(γ-glutamyl)spermidine, and lysine is seen, as is that of members of a second group that contains N^1-(γ-glutamyl)spermidine, N^8-(γ-glutamyl)spermidine, and hypusine.

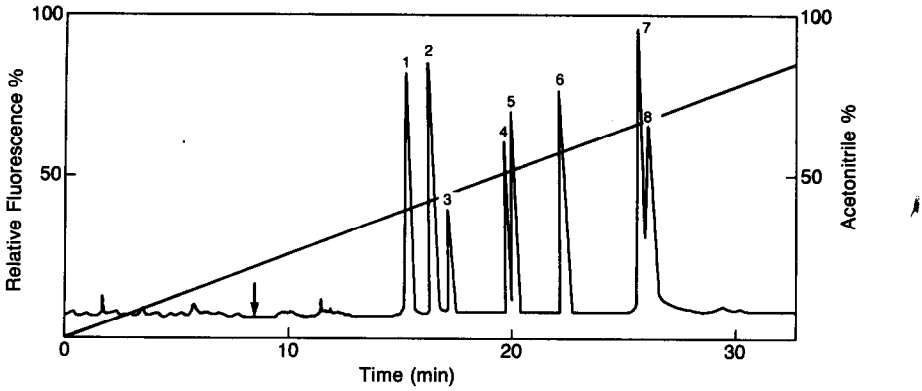

Fig. 1. Chromatographic pattern of the OPA derivatives of several γ-glutamylamines (*ca.* 0.3 nmol of each), lysine (1 nmol), and hypusine (0.1 nmol). The vertical arrow indicates the approximate point at which the elution of all salts that are carried over from ion-exchange chromatography and the derivatization procedure is completed. The peaks are those of the derivatives of N^1,N^8-bis(γ-glutamyl)spermidine (1), N^1,N^4-bis(γ-glutamyl)putrescine (2), ε-(γ-glutamyl)lysine (3), N^1-(γ-glutamyl)sperdimine (4), N^8-(γ-glutamyl)spermidine (5), N-(γ-glutamyl)putrescine (6), lysine (7), and hypusine (8).

When a standard mixture of amino acids (2- ml of amino acid standard H) was chromatographed in the ion-exchange system and the combined fractions in which the individual γ-glutamylamines are known to be eluted, were treated with OPA and aliquots of the treated combined fractions applied separately to the HPLC system, no peaks were observed at the positions of the γ-glutamylamine derivatives (data not shown). This separation of amino acid and γ-glutamylamine derivatives was confirmed by the observations that: (i) when this procedure was repeated with a mixture of amino acids and γ-glutamylamines, patterns were produced in the HPLC system that differed only in the occurrence of additional peaks for the γ-glutamylamine derivatives and (ii) that these additional peaks of γ-glutamylamine derivatives were completely absent when the γ-glutamylamines in the combined fractions from ion-exchange chromatography were destroyed by acid hydrolysis (6 *M* hydrochloric acid for 18 h at 110°C) prior to treatment of the fraction with OPA in preparation for HPLC.

The simple HPLC system described here was the most satisfactory found for complete resolution of γ-glutamylamines from other components of protein digests within a reasonable period of time, while providing good peak sharpness and symmetry. The gradient of acetonitrile, commencing at 0% at the time of sample application, was chosen in order to allow ample time for complete elution of salts carried over from ion-exchange chromatography and from the derivatization reaction before the start of elution of OPA derivatives (Fig. 1). Whereas the ion-exchange buffer salts sodium citrate and sodium chloride did not in any way interfere with OPA derivative formation, salts did cause pronounced changes in retention times and poor peak shapes of some OPA derivatives when eluted together with the derivatives. The gradient employed provides a means of avoiding a desalting step without involving a change in chromatographic performance and with little increase in the time needed for chromatography.

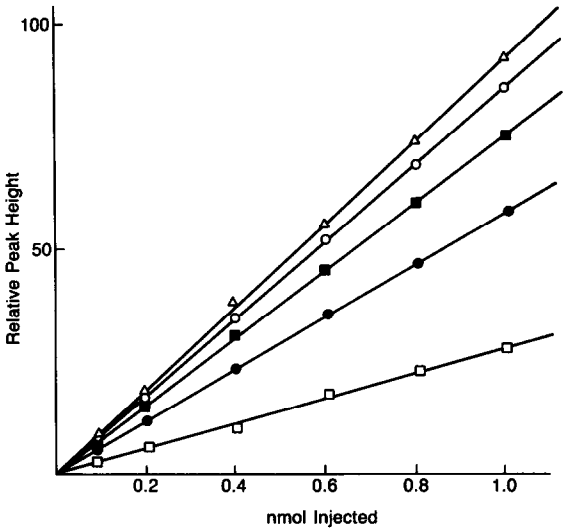

Fig. 2. Concentration–response relationship of the OPA derivatives of N^1,N^8-bis(γ-glutamyl)spermidine (△), N^1,N^4-bis(γ-glutamyl)putrescine (○), N-(γ-glutamyl)putrescine (■), N^1- and N^8-(γ-glutamyl)spermidines (●), and ε-(γ-glutamyl)lysine (□).

Linearity

Standardization curves for ε-(γ-glutamyl)lysine and for the γ-glutamylpolyamines displayed linear concentration–response relationships. The excellent consistency of peak shape allowed construction of curves with the use of peak-height response (Fig. 2). γ-Glutamylamines in amounts of 0.1–1 nmol were used for standardization, and regression analyses indicated no significant deviation from linearity with any substrate.

Application and sensitivity

The method has been used for determination of the level of γ-glutamylamines in epidermal cell envelopes from the skin of normal individuals and of psoriatic patients[13] and from the skin of the newborn mouse. The HPLC chromatogram obtained for OPA derivatives of components in various combined fractions from the ion-exchange chromatogram of a proteolytic digest of cell envelope from newborn mouse epidermis are shown in Fig. 3. The sensitivity of the method was evaluated by estimating the limits of detection from the results obtained with the mouse cell envelope. The detection limits were observed to be approximately 10 and 200 pmol/mg of protein for γ-glutamylpolyamines and for ε-(γ-glutamyl)lysine, respectively, based on a signal-to-noise ratio of 3.

Derivative production

It has been reported that the stability of OPA derivatives of amines and amino acids is influenced by a number of experimental parameters, including OPA concentration[14,15], the thiol compound used as coreactant[15–18], amine structure[15], water

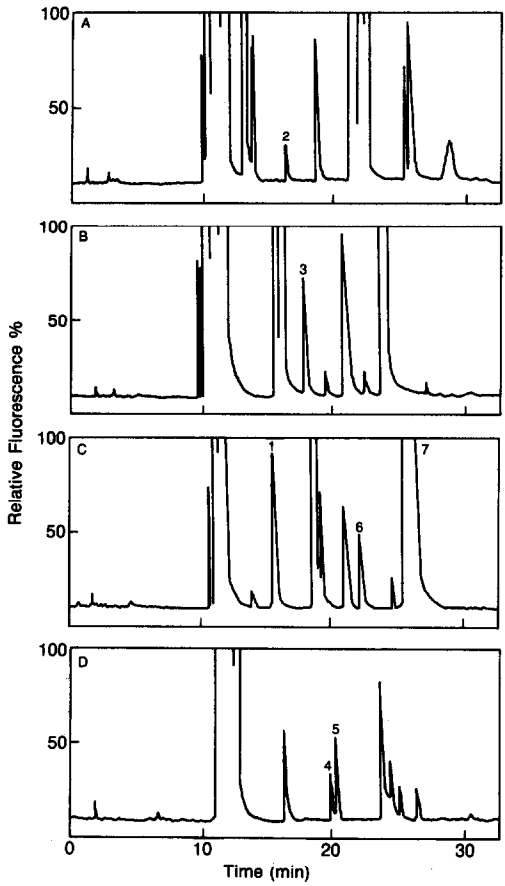

Fig. 3. Chromatographic patterns obtained upon analysis of mouse epidermal cell envelope for γ-glutamylamines. The cell envelope (10 mg) which remains after exhaustive extraction of epidermis with a buffered solution of urea, sodium dodecyl sulfate, and mercaptoethanol[13] was digested with proteolytic enzymes[4] and the digest was chromatographed in an ion-exchange system, as previous described[10] with collection of 1-ml fractions. Combined fractions were treated with OPA as outlined, and appropriate aliquots (0.15–1.2 ml) of the mixture of OPA derivatives were injected into the HPLC system. The numbers explained in the legend to Fig. 1 identify the derivatives. (A) Combined fractions containing compound 2; aliquot equivalent to 1.63 mg of epidermal cell envelope injected. (B) Combined fractions containing compound 3; aliquot equivalent to 0.375 mg of epidermal cell envelope injected. (C) Combined fractions containing compounds 1 and 6; aliquot equivalent to 0.375 mg of epidermal cell envelope injected. (D) Combined fractions containing compounds 4 and 5; aliquot equivalent to 3 mg of epidermal cell envelope injected.

content[15], and pH, both during[15] and following[19] formation of the derivatives. Of these, the level of OPA used for derivatization is believed to be most critical[14,15], and the use of large excesses of OPA is discouraged. The amount of OPA in the reagent employed here for the pre-column reaction was selected on the basis of trials and was optimized for rapid and near-quantitative derivative production in the present application with fractions from the ion-exchange chromatogram of the digest from *ca.* 10 mg of cell envelope. However, the use of this reagent also provided

optimal derivatization for preparation of standardization curves, even when used at very low concentrations of γ-glutamylamines (1–10 μ*M*), if the conditions for the reaction were followed closely and injections into the HPLC system were made immediately upon derivatization. Thus, with close adherence to these conditions, the present procedure should be generally applicable to samples of widely varying substrate concentrations.

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