High Performance Liquid Chromatography Analysis of Amino Acids at the Picomole Level[1]

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ABSTRACT

A method was developed for analyzing the amino acid compositions of protein hydrolysates by high performance liquid chromatography (HPLC) in less than 1 hr. Ten picomoles of each of the commonly occurring α-amino acids could be reliably determined by the fluorescence of their *o*-phthaldialdehyde (OPA) derivatives. The OPA-derivatization method, which uses ethanethiol as the sulfhydryl reagent, yields amino acid derivatives that are more highly fluorescent than OPA/mercaptoethanol derivatives and are stable for at least 2-1/2 hr. This HPLC method gives a better separation than previously published methods. Amino acid analysis down to the 3-pmol level is demonstrated, and problems associated with analyses at the low picomole level are discussed.

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A method has long been needed for quickly and reliably determining the amino acid compositions from microgram quantities of proteins. Standard amino acid analysis methods require from five to several hundred micrograms of purified protein, a 20-hr hydrolysis at 110° C (Moore and Stein 1963), and a 2-hr chromatographic analysis (Spackman et al 1958). Because many proteins, e.g., hormones and enzymes, are obtained in small quantities by such techniques as preparative isoelectric focusing and electrophoresis, a method for determining the amino acid compositions of proteins available in microgram to milligram amounts is required.

Trace levels of various fluorescent derivatives of amino acids have been determined by high performance liquid chromatography (HPLC) (Roth 1971, Roth and Hampai 1973, Engelhart et al 1974, Benson and Hare 1975, Bayer et al 1976, Zimmerman et al 1977, Bachmann et al 1979, Hill et al 1979, Rubinstein et al 1979, Spadero et al 1979, Shaw et al 1980, Jones et al 1981). Derivatization of α-amino acids with *o*-phthaldialdehyde (OPA) in the presence of a thiol reagent such as 2-mercaptoethanol (Roth 1971, Roth and Hampai 1973, Benson and Hare 1975, Shaw et al 1980, Jones et al 1981) or ethanethiol (Hill et al 1979, Lookhart et al 1982) yields highly fluorescent compounds that can be detected at picomole sensitivity. Precolumn (Hill et al 1979, Jones et al 1981, Lookhart et al 1982) and postcolumn (Roth 1971, Roth and Hampai 1973, Benson and Hare 1975, Shaw et al 1980) OPA derivatizations have been used to analyze amino acids hydrolyzed from microgram quantities of protein. Analysis times of from 35 min to 3 hr were required.

This paper reports a method for reliably analyzing quantities of 10 picomoles or more of all the common amino acids in 52 min. It also describes the fact that the OPA-amino acid derivatives formed in the presence of ethanethiol were more stable and more fluorescent than the analogous 2-mercaptoethanol derivatives. This method is superior to other published amino acid analysis methods because the analysis times are considerably reduced, and the resolution and stabilities of the amino acid derivatives are improved.

MATERIALS AND METHODS

Reagents and Chemicals

Water was deionized; all other solvents were of HPLC grade obtained from Burdick and Jackson Laboratories, Inc. Reagent grade sodium phosphate, OPA, boric acid, and 2-mercaptoethanol were obtained from Sigma Chemical Co. The amino acid standard calibration mixture was from Beckman Instruments Co.; and hydrochloric acid, ethanethiol, and S-β-(4-pyridylethyl)-L-cysteine (PC) were from Pierce Chemical Co.

Protein Hydrolysis

The protein hydrolysis procedure has been described in detail elsewhere (Lookhart et al 1982).

Amino Acid Derivatization

Protein hydrolysates and amino acid calibration mixtures were derivatized with OPA (Lookhart et al 1982) by a modification of the procedure of Hill et al (1979). Aliquots (5 μl) of the amino acid calibration mixture or protein hydrolysate containing from 2 to 50 pmol/μl of each amino acid were placed in small volume insert vials from Waters Associates (300 μl, catalog no. 72704), followed in order by 25 μl of deionized water, 25 μl of saturated sodium borate buffer (pH 9.5), 12.5 μl of OPA-derivatizing reagent, and 62.5 μl of methanol. The vial was thoroughly mixed on a vortex mixer after each addition. The mixture was normally kept at room temperature for at least 2 min (but not more than 10 min), after which a 50-μl aliquot was injected into the HPLC system.

The OPA-derivatizing reagent was stable up to one week and consisted of 25 mg of OPA, 50 μl of thiol reagent (2-mercaptoethanol or ethanethiol), 0.50 ml of saturated borate buffer (pH 9.5), and 4.5 ml of methanol.

Stability of OPA Derivatives Formed in the Presence of Different Thiol Reagents

Two derivatizing reagents were made up, one with ethanethiol, the other with 2-mercaptoethanol. Five-microliter aliquots of the amino acid standard mix (50 pmol/μl of each amino acid) were derivatized as discussed above. At various times (0.5–10 min) following addition of the derivatizing reagent, 50 μl of the 130-μl OPA-amino acid reaction solution were injected. The samples analyzed after long reaction periods (0.5–18 hr) were taken from a single large reaction mixture containing 10 times the volume of each individual reagent as listed for the short time periods and were injected by a Waters Associates WISP 710 A autosampler.

High Performance Liquid Chromatography

The HPLC system consisted of a Varian 5060 pump, Rheodyne 7120 injector (50-μl loop), one or two Waters Associates μ-Bondapak C-18 columns (each 30 cm × 3.9 mm i.d.) connected in series, a WISP 710 A autosampler, and a Turner Designs model 10 filter fluorometer. The fluorometer used a Corning 7-60 excitation filter (open between 310 and 390 nm) and two emission filters, a Wratten 2A and a Corning 4-96 (clear above 410 nm), as suggested by Turner Designs. The detector response was displayed and peak areas were calculated by a Hewlett-Packard 3388A printer-plotter automation system. The five-step multilinear gradient used (Table I) was a modification of that reported by Hill et al (1979) and

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Lookhart et al (1982) and was composed of acetonitrile, CH_3CN (solvent A), and 0.01 *M* Na_2HPO_4 at pH 7.4 (solvent B). The flow rate was 1.0 ml/min, and the back pressure was 2,300 psi. The method resolved the OPA derivatives of all the common α-amino acids (except cysteine) found in protein hydrolysates, as well as PC, cysteic acid, and carboxymethyl cysteine. Successive samples could be injected every 65 min.

RESULTS AND DISCUSSION

Amino Acid Analysis by HPLC

Chromatograms of a standard amino acid mixture (96 pmol of each amino acid) separated by passage through one column or two columns in series are shown in Figures 1 and 2, respectively. Using a single column and the gradient in Table I gave very poor separation of threonine (T) from glycine (G) while PC and isoleucine (I) coeluted. The gradient used for Figure 1 was more shallow than that used in Figure 2 (Table I). Even so, the best resolution achieved with a single column (Fig. 1) still left threonine and glycine partially resolved, as were PC and isoleucine. Near baseline separation of all OPA-amino acids (Fig. 2) was accomplished with dual columns using the gradient in Table I. Therefore, all other analyses were made with the dual column procedure. Each peak in the chromatograms (Figs. 1 and 2) corresponded to 96 pmol of amino acid, except the PC peak, which represents 960 pmol. The molar fluorescence of the OPA derivative of PC was only about 1/11 that of the other OPA-amino acid derivatives.

Histidine and arginine were the most sensitive to buffer pH and column loading effects. Whenever new columns were installed, it was sometimes necessary to adjust the buffer pH slightly to separate histidine (H) and arginine (R) from the other amino acids. The relative retention times of histidine and arginine were altered by a relatively small pH change; lowering the pH by 0.1 unit increased the retention times for histidine and arginine by about 0.5 min. The relative retention times of the other amino acids were not affected by pH changes between 6.5 and 7.5.

Since the OPA reagent does not react with *imino* acids, proline (P) and hydroxyproline could not be detected.

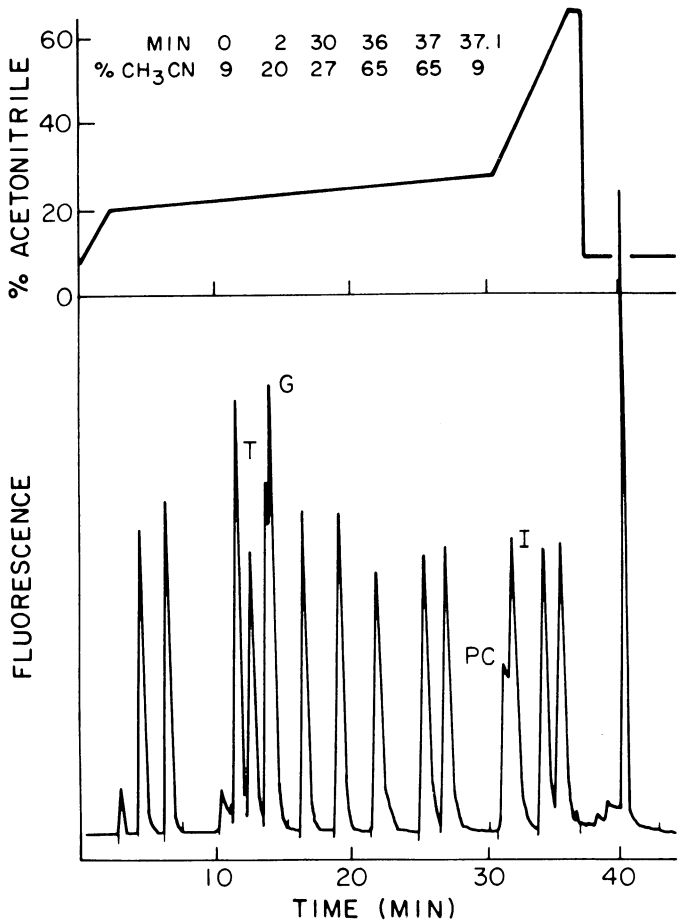


Fig. 1. Separation of *o*-phthaldialdehyde (OPA)-amino acid derivatives with a single C_{18} column, using an acetonitrile–0.01 *M* Na_2HPO_4 (pH 7.4) gradient. G = glycine, I = isoleucine, PC = pyridylethyl cysteine, T = threonine.

TABLE I
Multi-Linear Gradient for Separating *o*-phthaldialdehyde (OPA)-Amino Acids by High Performance Liquid Chromatography[a]

	Time (min)					
Solvent	**0**	**2**	**35**	**55**	**56**	**56.1**
% A, CH_3CN	9	20	32	80	80	9
% B[b]	91	80	68	20	20	91

[a]The program consisted of a series of three linear gradients, an isocratic hold, and a final linear gradient to return the column to initial conditions.
[b]Solvent B was 0.01 *M* Na_2HPO_4, pH 7.4.

Stability of Derivatives

The stability and molar fluorescence intensity of the OPA-amino acid derivatives depended on which thiol reagent, 2-mercaptoethanol (2-ME) or ethanethiol (ET), was present in the reaction mixture. The stabilities of the OPA-amino acids formed in the presence of 2-ME are shown in Figures 3–6. Aliquots of the amino acid mixture were reacted, incubated from 0.5 to 10 min (Figs. 3 and 4) or from 0.5 to 12 hr (Figs. 5 and 6), and then analyzed. Two figures were made for each time period to reduce overlapping of data. The 2-ME OPA-amino acid derivatives were stable for incubation times between 2 and 10 min. Considerable variations were found in the molar fluorescence responses of individual amino acids in the first 2 min following mixing, as well as between amino acids (peak area counts ranged from about 500 for lysine and aspartic acid to 2,000 for isoleucine). Instability of most of the 2-ME OPA-amino acids was found when incubation periods

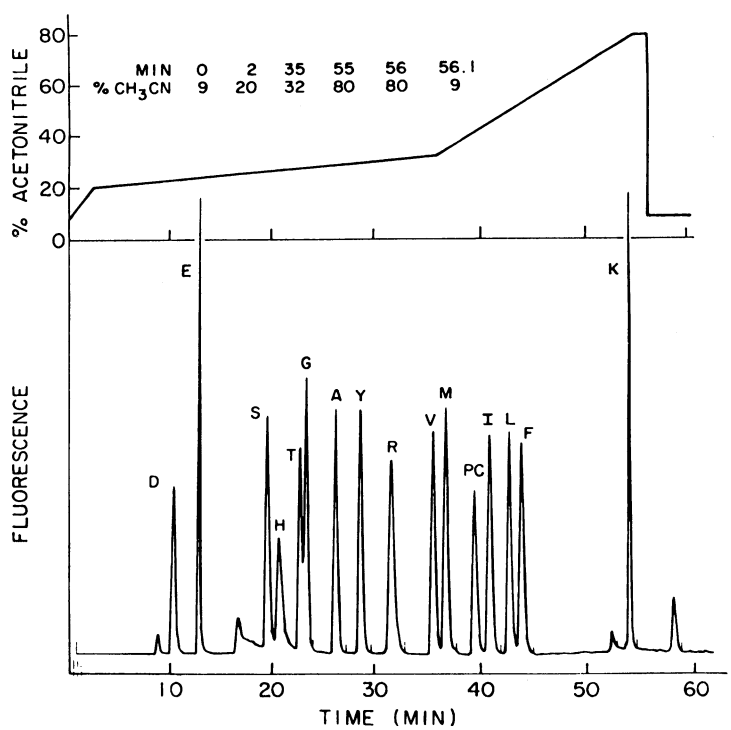


Fig. 2. Separation of *o*-phthaldialdehyde (OPA)-amino acid derivatives with two C_{18} columns in series, using an acetonitrile–0.01 *M* Na_2HPO_4 (pH 7.4) gradient. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

exceeding 30 min were studied. The fluorescence intensity of lysine (K) had dropped to about 10% of its initial value after 2 hr. The fluorescence intensities of the other amino acids dropped 5–10% in the first 2 hr after mixing, and only isoleucine (I), valine (V), tyrosine (Y), phenylalanine (F), and PC were stable for 3 hr or longer. 2-ME is acceptable as a reducing agent only if the OPA-amino acids are injected into the HPLC within 2–10 min after mixing, and every sample must be injected at identical incubation times. Even within the 10-min limitation, the molar fluorescence responses, and hence ability to quantitate lysine (K) and aspartic acid (D), were only 1/2 to 1/4 that of the other amino acids.

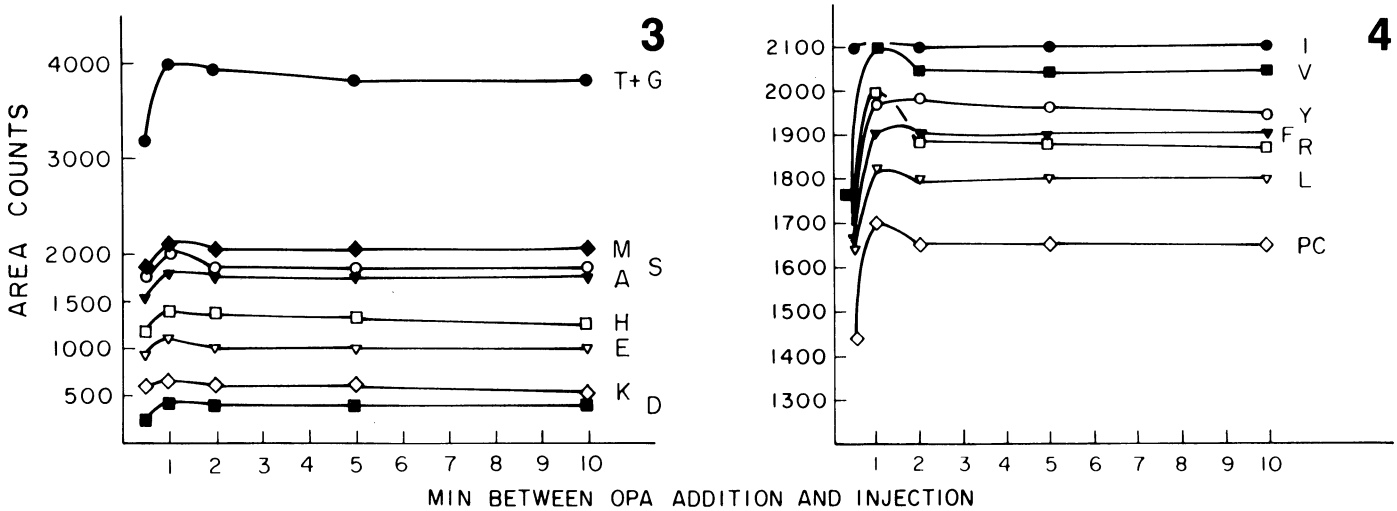
The stabilities and fluorescence intensities of OPA-amino acids formed utilizing ET as the sulfhydryl reagent are shown in Figures 7 and 8 (all were plotted relative to leucine = 10,000). All ET OPA-amino acid derivatives were stable for 10 min following mixing and had fluorescence intensities averaging seven times that of their 2-ME counterparts. Only threonine and glycine had significant (total 10% for both) reduction in fluorescence intensity 2 hr after mixing. The threonine and glycine ET-OPA data were combined for comparison with the 2-ME data. The ET-OPA derivatives of all the other amino acids (including lysine) were stable, a decrease in fluorescence of less than 5%, for at least 2 hr after mixing, and seven of them were stable for at least 18 hr. These facts corroborate the reports of Hill et al (1979) and Lookhart et al (1982) on using ET to give highly fluorescent and stable OPA-amino acids. For those reasons, ET was used as the reducing agent in forming the OPA-amino acid derivatives in this study.

Fluorescence Linearity with Concentration

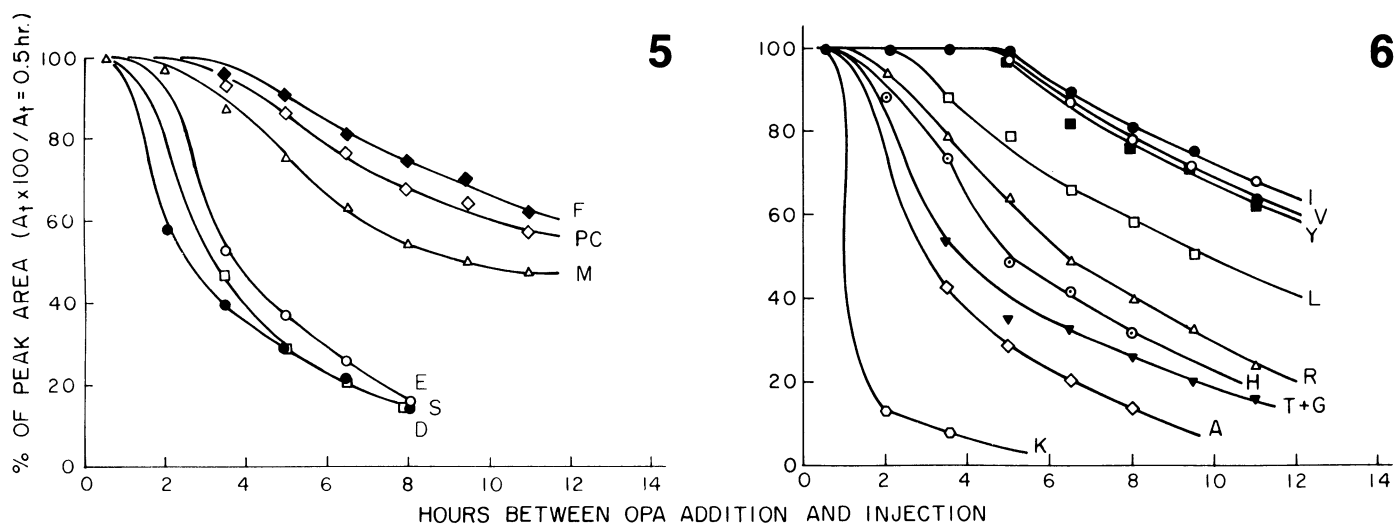
A linear relationship was found between the amounts of amino acids reacted and the fluorescence response (peak areas) of the ET OPA-amino acids in the concentration range between 80 and 800 pmol (Figs. 9 and 10). This relationship held up to a concentration of at least 2,500 pmol, and was not tested above that point.

The slopes of the concentration versus fluorescence lines for the different amino acids vary, indicating they have different "molar fluorescence coefficients." For example, 1 pmol of OPA-arginine complex fluoresces more strongly than does 1 pmol of OPA-threonine complex. Most of the OPA-amino acid complexes exhibit somewhat similar fluorescence responses. The molar fluorescence response of the OPA-PC complex, however, was about 1/11 that of the other complexes, as mentioned earlier.

The linear relationships found between the fluorescence



Figs. 3-4. Stability of 2-mercaptoethanol *o*-phthaldialdehyde (OPA)-amino acid derivatives analyzed from 1/2 to 10 min after mixing. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.



Figs. 5-6. Stability of 2-mercaptoethanol *o*-phthaldialdehyde (OPA)-amino acid derivatives analyzed from 1/2 to 12 hr after mixing. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

responses of each OPA-amino acid and concentration in the range from 3 to 80 pmol are shown in Figures 11 and 12. Figure 11 shows the relationship for 12 of the OPA-amino acid complexes, and four others are shown in Figure 12. Eight of the OPA-amino acid complexes gave almost identical fluorescence responses with concentration, and those are plotted in the shaded areas. Straight line relationships were found between amino acid concentration and OPA-amino acid fluorescence peak areas for all amino acids except histidine in the 10 to 80 pmol range. All except one of the lines extrapolated back to the origin (0 fluorescence at 0 concentration). For the OPA-histidine complex, there was a straight line relationship between peak area and histidine concentration above 20 pmol, but the line did not extrapolate back to zero. The OPA-histidine peak area values were not integrated correctly for concentrations of histidine less than 10 pmol because of the low relative fluorescence of the complex, and because the peak did not elute sharply.

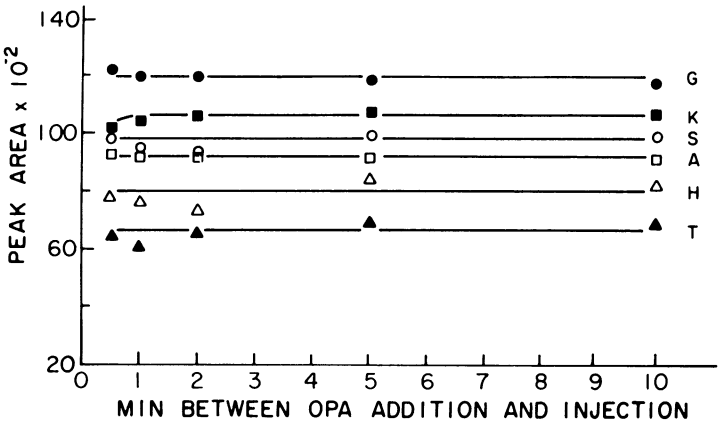


Fig. 7. Stability of ethanethiol *o*-phthaldialdehyde (OPA)-amino acid derivatives analyzed from 1/2 to 10 min after mixing. A = alanine, G = glycine, H = histidine, K = lysine, S = serine, T = threonine.

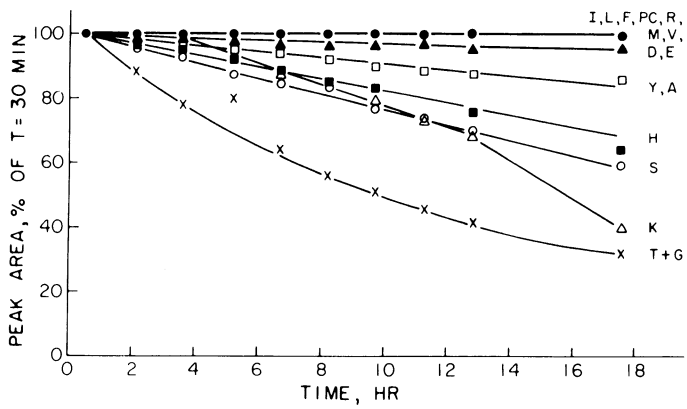


Fig. 8. Stability of ethanethiol *o*-phthaldialdehyde (OPA)-amino acid derivatives analyzed from 1/2 to 18 hr after mixing. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

The fluorescence response of the OPA-serine peak was larger than the fluorescence response line predicted for serine amounts of 10 pmol or less, which implied that more serine was present than was added to the reaction mixture.

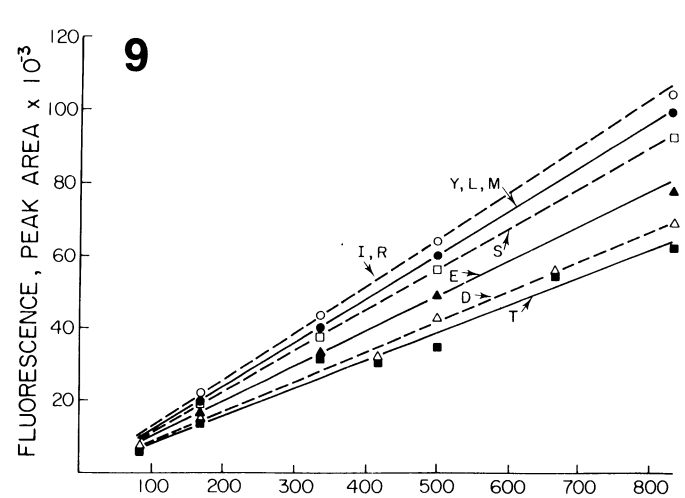
The PC line shown has 10 times more material at each point than is indicated on the x-axis. Because of the low molar fluorescence of the OPA-PC complex, it cannot be reliably determined when present in amounts less than 80 pmol.

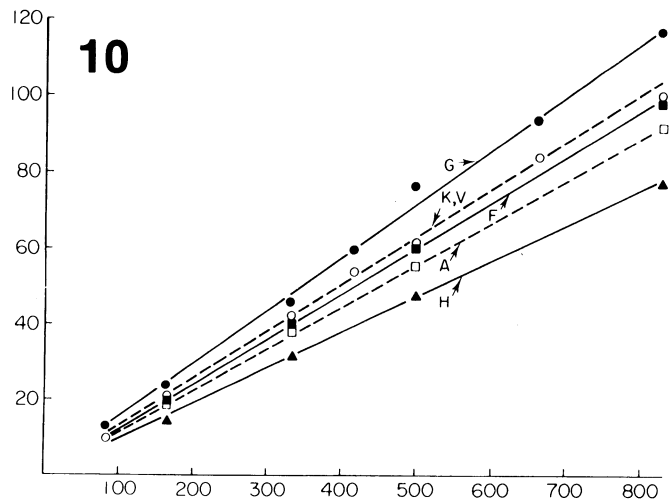
The relationship of the fluorescence peak areas to the amount of the OPA complexes of glycine (G), lysine (K), aspartic (D), and glutamic acids (E) are shown in Figure 12. They can all be reliably assayed at the 15 pmol and higher levels. However, the fluorescence responses for amounts of OPA-glycine and OPA-lysine at less than 15 pmol were higher than the extrapolated line relationship predicted. The reason for the higher-than-expected fluorescence values for very low amounts of OPA-glycine and OPA-serine was found by chromatographing a 5-μl sample of water containing no added amino acids (Fig. 13). Very small peaks were found at the position where OPA complexes of serine, glycine, and alanine normally elute. Minute amounts (<1 pmol) of those amino acids must have been present in the water, derivatization mixture, or on the glassware. Those peaks could possibly be caused by other compounds that react with OPA and elute at these positions. However, they are only of significance when analyzing samples at the 3–10 pmol level.

An OPA-amino acid chromatogram resulting from the reaction of 3 pmol of each amino acid is shown in Figure 14. The integrator attenuation of the peaks is the same as that used for the water blank in Figure 13, and it shows the relative effect of the trace contaminants on amino acid analyses at the 3-pmol level. It is obvious from Figures 13 and 14 that water blanks must be run and consideration made for trace components when analyzing OPA-amino acids at the low picomole range.

Analysis of Protein Hydrolysates

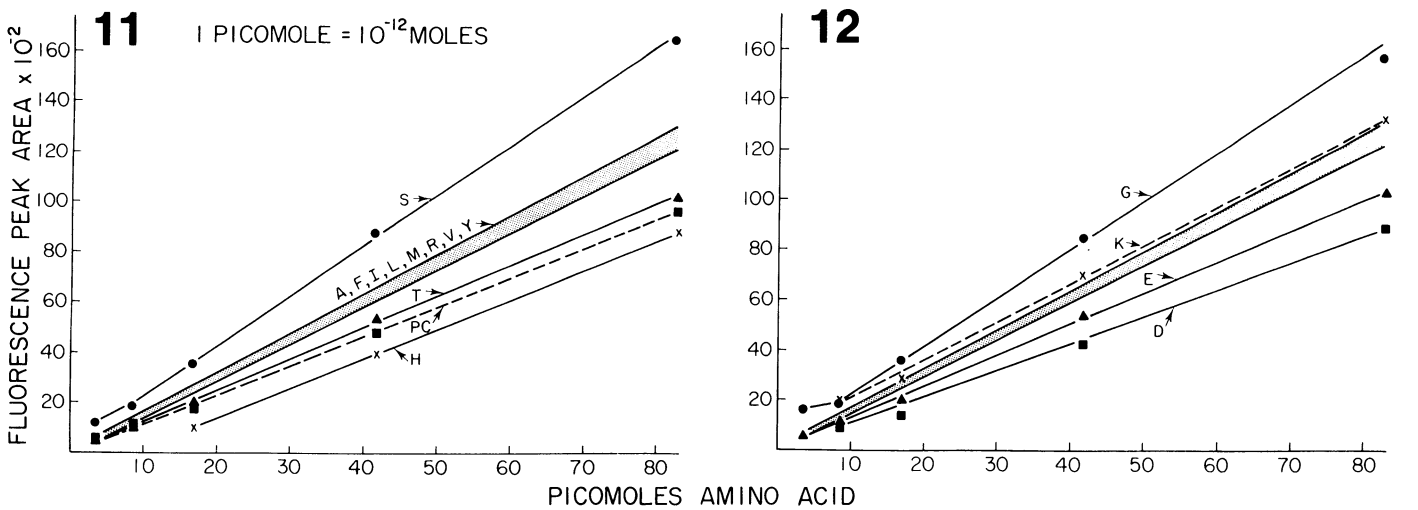
When 20 pmol of a protein (β-purothionin) was hydrolyzed and





PICOMOLES AMINO ACID

Figs. 9-10. Plots of fluorescence versus amount of amino acid derivatized by the ethanethiol *o*-phthaldialdehyde (OPA) method. Amino acids present in amounts from 80 to over 800 pmol. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = lysine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.



Figs. 11-12. Plots of fluorescence versus amount of amino acid derivatized by the ethanethiol *o*-phthaldialdehyde (OPA) method. Amino acids present in amounts from 3 to over 80 pmol. The shaded area in each figure contains amino acids A, F, I, L, M, R, V, and Y. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = lysine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

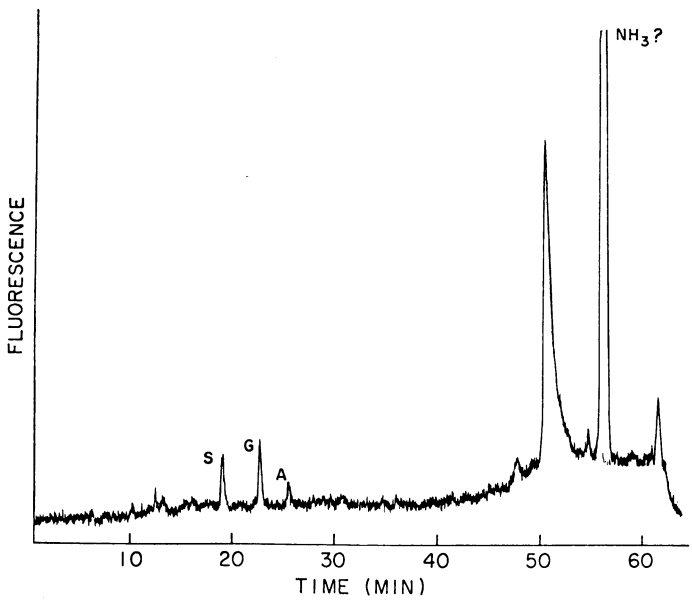


Fig. 13. Analysis of a 5-μl water blank containing ethanethiol *o*-phthaldialdehyde. Attenuation is the same as for 3-pmol analysis (Fig. 14). A = alanine, G = glycine, S = serine.

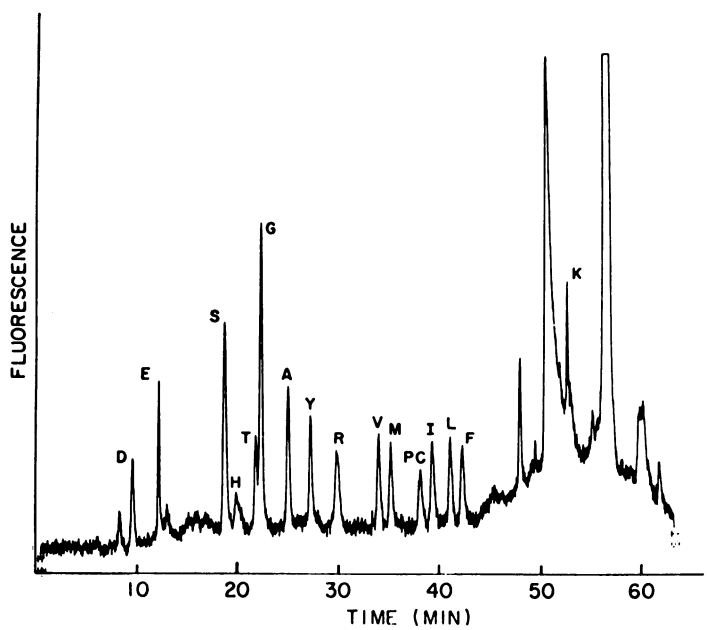


Fig. 14. Analysis of a sample containing 3 pmol of each amino acid (PC at 30 pmol), using dual columns and gradient as in Fig. 2. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = lysine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

the resulting hydrolysate analyzed by this OPA method, the results shown on the top of Figure 15 were obtained. The sensitivity of this method is obvious, since the analysis involved dissolving 1 mg of protein in 100 ml of water and then removing one ten-thousandth of it (10 μl) for hydrolysis and analysis. The peaks in the chromatogram (Fig. 15) were sharp and qualitatively consistent with sequence data. When the amino acid composition of the protein was calculated from the data, however, it differed slightly from the known composition. The lower half of Figure 15 shows the result obtained when a blank sample was "hydrolyzed" with HCl following the procedure used to hydrolyze the β-purothionin. This HCl blank chromatogram was obtained when 250 μl of HCl was sealed in an ampoule, heated, dried, and analyzed by the method of Lookhart et al (1982). The trace amounts of amino acids present in the HCl were the reasons for the differences found between the analyzed and known compositions of β-purothionin. The HCl contained small amounts of several amino acids, especially glutamic acid, serine, glycine, and alanine. Serine, glycine, and alanine were also found in the water blank, but at much lower levels (Fig. 13). The level of amino acids demonstrated in this HCl blank is up to 10 times the contamination that would be seen in a normal analysis, since only about 1/10 of the hydrolysate is normally reacted and analyzed. Several ultra-pure grade HCl samples from different sources were analyzed, and all contained significant amounts of amino acids for use in hydrolysis at the low picomole level.

In summary, an HPLC method for determining the amino acid compositions of protein samples down to the level of 3 pmol of each amino acid has been presented. However, when working with amounts of material less than about 100 pmol, appropriate controls must be run to compensate for the minute amounts of amino acids found in even the best of commercially available reagents.

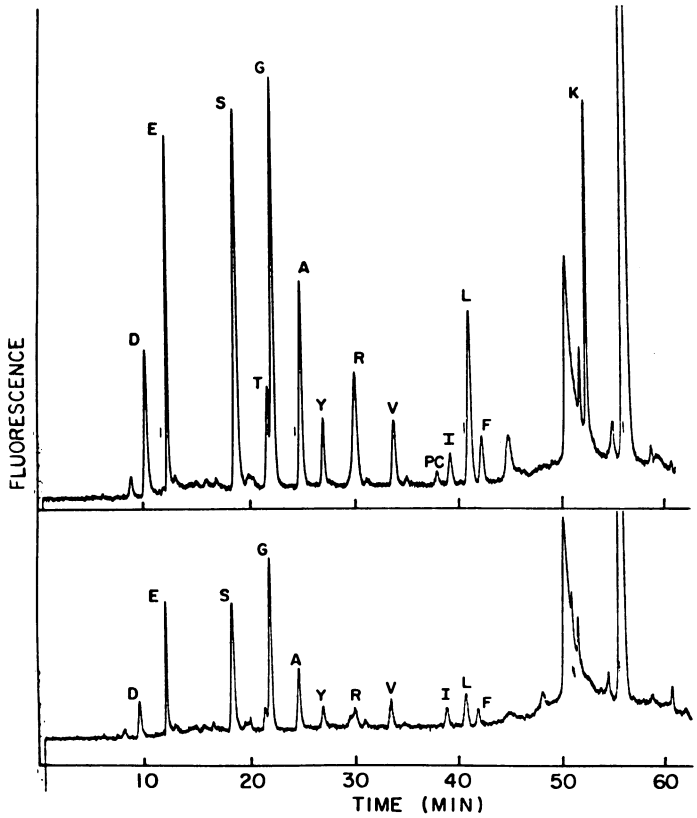


Fig. 15. Amino acid analyses of 20 pmol of hydrolyzed β-purothionin (top) and a 250-μl HCl blank (bottom). Both were analyzed using the columns and gradient of Fig. 2. A = alanine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = lysine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, PC = pyridylethyl cysteine, R = arginine, S = serine, T = threonine, V = valine, Y = tyrosine.

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