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Analysis of amino acids by liquid chromatography after pre-column derivatization with 4-nitrophenylisothiocyanate

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ABSTRACT

Derivatization of amino acids with the phenylisothiocyanate analogue, 4-nitrophenylisothiocyanate, results in stable thiocarbamyl derivatives which are suitable for analysis via liquid chromatography. Detection limits of *ca.* 1 pmol can be achieved with detection at either 254 or 340 nm. Separation of the normal hydrolyzate amino acids is readily accomplished in less than 25 min using a 30-cm reversed-phase column. Using reaction conditions optimized for derivative yield and minimal reagent interference, the procedure provided reproducible, accurate compositional analyses of hydrolyzed peptide and protein samples.

INTRODUCTION

Improved procedures for pre-column derivatization of amino acids have been the major driving force behind recent advancements in amino acid analysis. Fluorescent reagents such as 9-fluorenylmethyl chloroformate (FMOC)[1], 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F)[2] and *o*-phthalaldehyde[3] (OPA) have allowed femtomole (10^{-15}) and even attomole (10^{-18}) detection limits by liquid chromatography or capillary zone electrophoresis. Highly absorbing colored derivatives such as those found with 4-dimethylaminoazobenzene-4-sulfonyl (dabsyl) chloride[4] have also been employed to provide low picomole level analysis. One of the more popular procedures has utilized the sequence reagent phenylisothiocyanate (PITC) to form stable phenylthiocarbamyl (PTC) derivatives[5,6]. The isothiocyanate reacts with both primary and secondary amines (a major drawback of OPA[7]) and shows excellent selectivity towards the amine functionality. Reactions of derivatizing reagents with phenolic or imidizole side chains can cause problems with the other reagents but have not been reported for PITC.

Over the past seven years our laboratory has exploited the reaction selectivity of PITC to develop fast, accurate methods for a wide variety of samples including protein hydrolyzates[5], feed samples[8] and physiologic fluids[6]. Detection limits of 1 pmol[5] were achieved using 254-nm detection for the highly absorbing PTC derivatives. Thus, PITC derivatization provides sufficient sensitivity for 100-ng protein samples[9], below

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which level interference from environmental contamination usually precludes accurate analysis even with inherently more sensitive fluorescent tagging reagents.

Recently we have begun to study alternative isothiocyanate derivatives that would potentially offer the same advantages of PITC (*e.g.* selective, rapid reaction with primary and secondary amines) but improve either the sensitivity or detection selectivity. One of these reagents, 4-nitrophenylisothiocyanate (NPITC), has been shown to retain many of the favorable characteristics of PITC, and also allow selective detection at 340 nm. This paper discusses the use of NPITC for amino acid analysis, the first PITC analogue employed successfully for pre-column derivatization amino acid analysis.

MATERIALS AND METHODS

Materials

Amino acids, peptides and proteins were from Sigma (St. Louis, MO, U.S.A.). HPLC-grade acetonitrile was purchased from J T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade water was obtained from a Milli-Q® system (Millipore, Bedford, MA, U.S.A.) fed with a reverse osmosis purified supply. Constant-boiling HCl and triethylamine (TEA) were from Pierce (Rockford, IL, U.S.A.). Crude NPITC was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Other chemicals were reagent grade.

NPITC Purification

Crude NPITC was a gummy, orange solid. A 1-g amount of this material was purified by vacuum sublimation. The sample was heated to 96°C and the pressure was 0.13 mm. Approximately 800 mg (80% yield) of a free flowing, pale yellow powder with a sharp melting point at 106.5°C (lit. 107°C)[10] was recovered.

Derivatization

A 50-m*M* stock solution of NPITC in acetonitrile was prepared daily. The working reagent consisted of 490 μl of stock NPITC, 50 μl of 10% TEA in acetonitrile, and 50 μl of water. Amino acid standards were vacuum dried in 50 × 6 mm Pyrex® tubes. Derivatization was initiated by pipetting 10 μl of working reagent into the tubes following by vigorous vortexing. After 10 min, 40 μl of water were added, and excess reagent was removed by a single extraction with 50 μl of hexane, Freon®, ethyl acetate or toluene. Aliquots of 1–10 μl of the aqueous layer were injected for analysis. For extraction studies, 1–2 μl of the organic layer were analyzed.

Separation of NPITC amino acids

The chromatographic system consisted of two M510 pumps, a M712 WISP® autosampler, controlled by an M840 chromatography and data station. Detection was accomplished with a M490 multiwavelength detector or an M440 single-wavelength detector equipped with either a 254 or 340 nm filter kit. Spectra of derivatized samples and NPITC were collected with a 990+ photodiode array detector using a W600 solvent delivery system for the analysis. Column temperature was controlled to 46.0 ± 0.1°C with a temperature control module (all hardware from Waters Chromatography Division of Millipore).

Separations were carried out on a Pico · Tag® free amino acid analysis column (300 × 3.9 mm) from Waters using a linear gradient running from 85% A [94% 0.14 *M* sodium acetate, 0.05% TEA (v/v), pH adjusted to 6.4 with acetic acid, 6% acetonitrile] and 15% B (60% acetonitrile in water) to 40% A and 60% B in 20 min. The flow-rate was 1.0 ml/min. After a 5-min wash with 100% B, the column was equilibrated for 12 min under the initial conditions. Total analysis time (injection-to-injection) was 40 min. For extraction studies and spectral analysis the gradient was extended to 30 min ending at 80% B, 20% A.

Sample hydrolysis

Peptides and proteins were dissolved in water (1–5 mg/ml) and aliquots containing 1–5 μg were vacuum dried in 50 × 6 mm Pyrex tubes. Constant-boiling HCl (200 μl) was added to the bottom of the large vial containing the tubes and the samples were sealed under vacuum after three alternate vacuum-nitrogen purging steps. Hydrolysis was carried out at 112°C for 20–24 h, after which the samples were cooled, vacuum dried, and derivatized as described for standard solutions.

RESULTS

Extraction studies

Unlike PITC, NPITC cannot be easily removed under reduced pressure. The hydrophobic nature of isothiocyanates made solvent extraction a potential alternative to vacuum drying. Hexane (which has been used for PITC extraction[11]), Freon, ethyl acetate and toluene were compared for extraction efficiency. The non-polar solvents hexane and Freon were not as effective as the more polar solvents in removing NPITC. In contrast, ethyl acetate was very effective at reagent removal, but also extracted some of the hydrophobic derivatized amino acids. Extraction with toluene gave an acceptable compromise between reagent removal and recovery of the amino acid analytes. Subsequent experiments all used toluene for extraction.

Reaction optimization

Derivatization of a standard amino acid mixture with PITC is typically carried out using reagent concentrations of 0.5–1.0 *M*. Concentrations $\leqslant$ 300 m*M* can result in less than quantitative yields, particularly for Asp and Glu. In contrast to PITC, NPITC derivatization using 1 *M* reagent results in an enormous artifact peak that is only partially extracted by toluene. Limiting the reagent concentration to 50 m*M* eliminated the great majority of the artifact, and separation of the resulting smaller peak from the NPTC-amino acids was readily accomplished (Fig. 1A). However, with $\leqslant$ 25 m*M* reagent, reduced yields of amino acids were observed.

Chromatography of NPTC-amino acids

The derivatization products of NPITC are the nitro analogues of the PTC-amino acids formed from the reaction with PITC. Consequently, the chromatographic behavior of the NPTC-amino acids is very similar to that observed for PTC-amino acids. The effect of operating parameters such as ionic strength, pH, gradient steepness and column temperature are very similar to that reported for PTC-amino acids[6]. Thus, higher ionic strength increased retention of neutral and acidic amino acids without

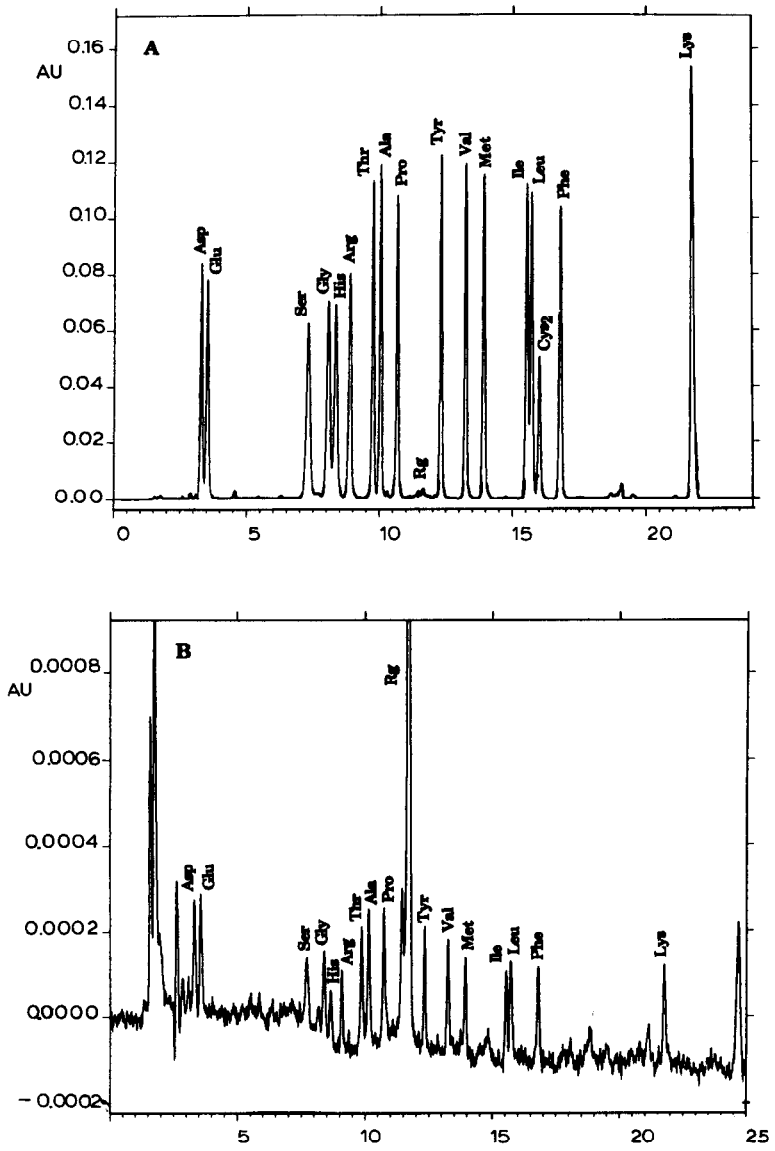

Fig. 1. Separations of NPITC-derivatized amino acids: (A) 833 pmol standard (B) 1.3 pmol standard. Full scale deflections are (A) 0.16 AU, (B) 0.001 AU. Horizontal axis: retention time in min. Chromatographic conditions and sample preparation are described in Materials and Methods.

affecting retention of basic derivatives. In fact, with only changes in gradient shape and initial solvent strength, resolution of all 18 amino acids in a standard mixture was readily accomplished (Fig. 1A) using identical mobile phases and the same column used for PTC-amino acid chromatography. The order of elution was the same except that Cys_2 elutes after Leu instead of before Ile. The optimized conditions also provide

TABLE I

REPRODUCIBILITY OF NPITC-AMINO ACID ANALYSIS

Based on five replicate analyses. C.V. = Coefficient of variation = 100 · standard deviation/average.

Amino acid	*C.V. (%)*	
	Peak area	*Retention time*
Asp	5.6	0.35
Glu	7.2	0.33
Ser	1.2	0.20
Gly	2.6	0.21
His	2.1	0.17
Arg	3.3	0.09
Thr	1.1	0.06
Ala	2.5	0.05
Pro	1.8	0.08
Tyr	0.7	0.07
Val	1.4	0.05
Met	1.5	0.04
Ile	1.4	0.05
Leu	1.9	0.03
Cys	22.6	0.04
Phe	1.5	0.05
Lys	5.9	0.04

TABLE II

COMPOSITIONAL ANALYSIS OF HYDROLYZED SAMPLES

Samples were prepared as described in the Materials and Methods section. Amounts injected were approximately 60 ng per sample. Expected values are given in parentheses.

Amino acid	*Lysozyme*	*Neurotensin*	*Angiotensin II*
Asp	20.0 (21)	1.0 (1)	0.9 (1)
Glu	5.8 (6)	1.9 (2)	0
Ser	8.1 (10)	0	0
Gly	12.1 (12)	0	0
His	0.7 (1)	0.1 (0)	1.1 (1)
Arg	11.0 (11)	2.5 (2)	1.1 (1)
Thr	6.5 (7)	0	0
Ala	12.4 (12)	0	0
Pro	2.4 (2)	2.0 (2)	1.1 (1)
Tyr	2.5 (3)	0.9 (2)	0.6 (1)
Val	5.4 (6)	0	1.0 (1)
Met	1.3 (2)	0	0
Ile	5.4 (6)	0.9 (1)	1.0(1)
Leu	7.9 (8)	1.9 (2)	0
Phe	3.1 (3)	0	1.1 (1)
Lys	5.8 (6)	0	0

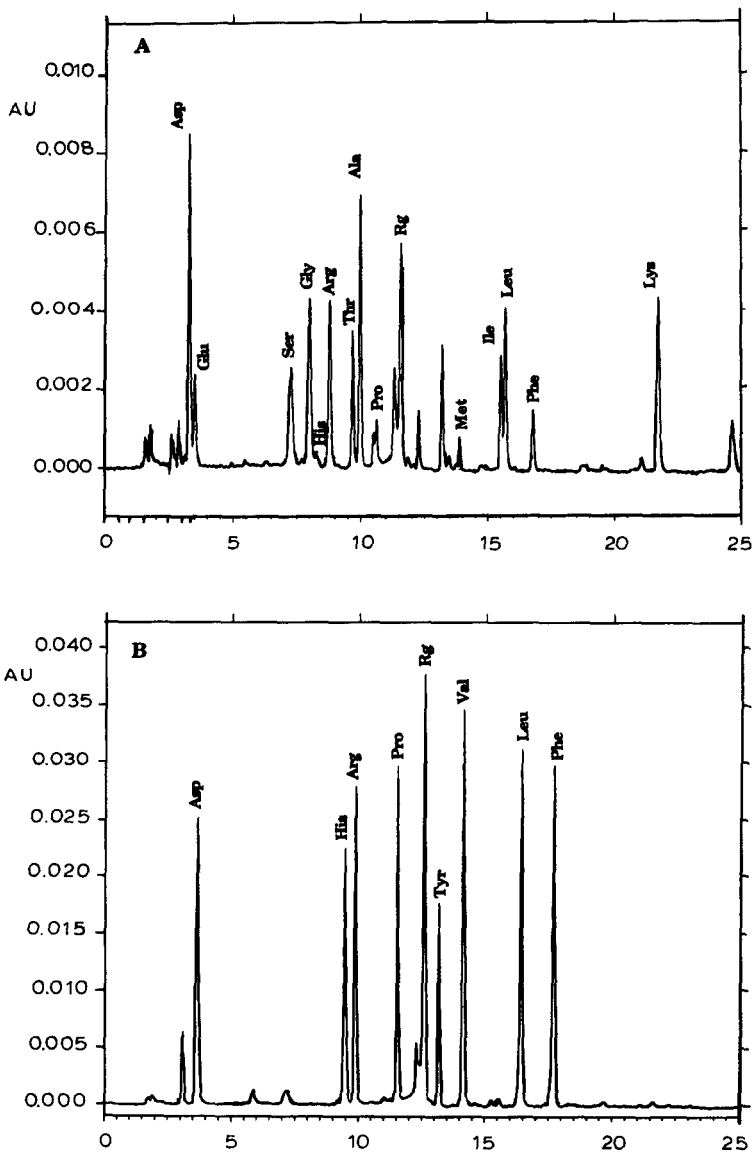

Fig. 2. Analysis of hydrolyzed (A) lysozyme and (B) angiotensin II. Conditions as in Fig. 1 except (A) AUFS = 0.01 and (B) AUFS = 0.04.

excellent resolution of the amino acid derivatives from the two major reagent peaks. Even at very low levels reagent interference is not observed. In general, the NPTC derivatives exhibit greater retention that their PTC analogs, an effect that is enhanced with the diderivatized compounds Lys and Cys_2.

Spectral analysis of eluting peaks with a photodiode array detector showed a peak maximum for NPITC at 335 nm with local maxima at 212 and 239 nm.

Carbamylation increased the relative absorbance of the second local maximum such that the NPTC derivatives exhibited absorption maxima at 236 nm, the longer wavelength maxima ranging from 341–347 nm. Absorbance was strong at 254 nm and detection limits of 0.5–1.0 pmol were estimated using UV detection at this wavelength (Fig. 1B) with a signal-to-noise ratio of 3. This is comparable to that reported for PTC-amino acids[5].

Reproducibility

Six replicate samples containing 12.5 nmol of each amino acid were derivatized and 5 μl out of a total of 75 μl aqueous layer were injected (833 pmol). Table I shows that the relative standard deviations ranged from approximately 1 to 7%, except for Cys_2, which was 23%. The reason for this large deviation has not been determined.

Protein and peptide compositional analysis

Protein and peptide samples (approximately 1 μg) were hydrolyzed with HCl, and aliquots containing 40–200 ng analyzed. Typical hydrolyzate samples are shown in Fig. 2. Compositions experimentally determined for the peptides neurotensin and angiotensin and the protein lysozyme agree very well with the expected values (Table II).

DISCUSSION

In a study on isothiocyanate sequencing reagents, Tarr[12] has recently reported on the reactivity of NPITC with peptides. Although found unsuitable as an alternative to PITC for protein/peptide sequencing, the rate of NH_2-terminal labelling with NPITC was shown to be more rapid than with PITC. The greater electrophilicity of NPITC is also the likely cause of large reagent related peaks that are observed in reactions where the NPITC concentration $\geqslant$ 200 m*M*. Side reactions with water and/or ethanol[13] are the most likely source of these peaks. However, greater reactivity allows the reagent concentration to be reduced more than 10-fold in comparison to standard PITC labelling procedures, thus reducing the reagent peaks to a manageable size.

Tarr also reported that NPITC-labelled peptides were slow to cyclize to the corresponding nitroanilino thiazolinone. Consistent with these findings are data from our lab showing markedly increased stability of the NPTC-amino acids in comparison to their PTC analogues. Thus, while slow cyclization is an unfavorable property for a sequencing reagent, the greater stability of NPTC compounds is very desirable for amino acid analysis.

Using the optimized reaction conditions, no evidence for side chain derivatization was observed with His or Tyr. Each of the amino acids yielded only a single peak, the retention being consistent with reaction at a single site (presumably the free amine). As expected, only Cys_2 and Lys formed disubstituted products. This simplifies the quantitation of these amino acids in comparison to more reactive compounds such as FMOC and NBD-F, which react with the imidazole of His[14] or the phenolic group of Tyr[15], respectively.

This report demonstrates that isothiocyanates besides PITC can be effective reagents for amino acid analysis via precolumn derivatization. Although most of the

data reported herein were obtained via detection at 254 nm, detection at 340 nm could prove very useful, as it is comparably sensitive, the increased absorbance and flatter baselines being offset by increased detector noise at the higher wavelength. Studies on samples such as urine, which contain components that absorb at 254 nm but not at 340 nm would benefit from detection at the higher wavelength and are currently in progress. While such components can pose potential interference problems with PITC analysis of urine amino acids[6], NPITC derivatization with derivative detection at 340 nm should eliminate most if not all interference from non-amine containing components. These studies also have provided guidelines for future work with isothiocyanate analogs. Rapid coupling to form thiocarbamyl derivatives is essential as is slow cyclization to the thiohydantoin. Analogues that are fluorescent or chemiluminescent are commercially available, and could allow the detection of subpicomole amounts of amino acids.

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