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Peptide maps at picomolar levels obtained by reversed-phase high-performance liquid chromatography and pre-column derivatization with phenyl isothiocyanate

Microsequencing of phenylthiocarbamyl peptides

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ABSTRACT

A new reversed-phase high-performance liquid chromatography approach to the production of analytical peptide maps by pre-column derivatization using phenylisothiocyanate is described. Tryptic peptide digests were derivatized with phenyl isothiocyanate to form the phenylthiocarbamyl peptides followed by reversed-phase high-performance liquid chromatographic analysis. The phenylthiocarbamyl peptides were separated by reversed-phase high-performance liquid chromatography with the conventional gradient elution system of water–acetonitrile containing trifluoroacetic acid. The sensitivity of detection of these peptide derivatives was within the range 5–10 pmol with a constant baseline at 254–260 nm. The isolated phenylthiocarbamyl peptides can be subjected to automatic Edman degradation. The effectiveness of this method was exemplified by microsequencing of phenylthiocarbamyl peptides isolated from tryptic digests of three different proteins: α-lactalbumin, β-lactoglobulin and a λ light-chain immunoglobulin.

INTRODUCTION

Phenyl isothiocyanate (PITC) has been the reagent most employed in the structural analysis of proteins since its introduction by Edman 40 years ago [1]. In the conventional Edman procedure for the sequential degradation of peptides or proteins, the free N-terminal amino acid reacts with PITC to form the phenylthiocarbamyl (PTC) derivative [2].

PITC, which reacts readily with amino acids [3], has also been used to quantify them at the picomolar level because it forms the PTC derivatives of the amino acids which can subsequently be identified and quantified by reversed-phase high-performance liquid chromatography (RP-HPLC) [4]. Various pre-column derivatization PTC

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systems for amino acid analysis of proteins and peptides [5,6], including one commercial PTC amino acid analyzer [7], as well as a derivatization of two synthetic peptides, dynorphyn and leucine-enkephalin, have been reported [8].

The present study describes a new approach, again using the Edman reagent PITC for the pre-column derivatization of peptide mixture digests, the separation of the resulting PTC-peptides by RP-HPLC with a detection sensitivity of the order of picomoles and the microsequence analysis of the isolated PTC-peptides.

EXPERIMENTAL

Acetonitrile was from Scharlau (Barcelona, Spain). Guanidinium chloride, iodoacetic acid and dithiothreitol were purchased from Sigma (St. Louis, MO, USA). PITC was from Beckman Instruments (Palo Alto, CA, USA). Triethylamine, methanol, tetrahydrofuran, 1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)–trypsin, trifluoroacetic acid and other compounds not specified were from Merck (Darmstadt, Germany). Ultrapure water for HPLC, generated by a Milli-RO4-coupled to a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used in the preparation of all buffers.

Proteins

A α-chain (L-chain) immunoglobulin (Ig) was obtained from a human (Escolano, ESC) monoclonal IgM after reduction and alkylation. Bovine α-lactalbumin and β-lactoglobulin were from Sigma.

Reduction and alkylation

Native proteins (3 mg) dissolved in 200 μl of 2 *M* Tris–HCl buffer, pH 8.6, containing 0.002 *M* EDTA and 6 *M* guanidinium chloride were incubated with 35 m*M* dithiothreitol for 120 min at 37°C. Alkylation was performed by adding 5.0 mg of idoacetic acid followed by incubation for 15 min at room temperature in the absence of light. The excess reagents were removed by gel filtration on a Sephadex G-25 column.

Trypsin digestion

Reduced and carboxymethylated proteins (500 μg) were digested with 5 μg of TPCK–trypsin in 100 μl of 0.2 *M* N-methylmorfoline acetate buffer, pH 8.2, for 1 h at 37°C. After digestion the material was freeze-dried.

Manual labeling of peptide mixtures with PITC

Tryptic digests (5–3000 pmol) were dried in Eppendorf tubes and dissolved in 15 μl of (methanol–water–triethylamine, 2:2:1) at room temperature. This solution was evaporated to dryness by rotary evaporation under high vacuum for 30 min. This step was repeated three times. The dry peptides were dissolved in 20 μl of methanol–triethylamine–water–PITC (80:10:10:1). After 30 min reaction at room temperature, the solution was evaporated to dryness under high vacuum for 40–50 min. The resulting PTC-peptides were dissolved in 6 *M* guanidinium hydrochloride containing 0.1% trifluoroacetic acid (TFA) and either injected into the RP-HPLC system or kept in the freezer for 1–10 days prior to being used for subsequent RP-HPLC analysis.

RP-HPLC separation of PTC-peptide mixtures

The chromatograph consisted of two Waters M6000A pumps, a Waters 680 automated gradient controller and a Waters 990 photodiode array detector with a dynamic range from ultraviolet to the visible (UV–VIS) region (190–800 nm), based on an NEC APC III personal computer. All sample injections were made with a Waters U6K universal injector. Separations were made by RP-HPLC on a Nova-Pak column (15 cm I.D. × 3.9 mm) protected by a guard column packed with μBondapak C_{18}–Corasil. The column was eluted with acetonitrile gradients containing 0.1% TFA. The column was operated at room temperature at a flow-rate of 0.5 ml/min. The PTC peptides were collected, lyophilized and kept at 4°C before further sequence analysis.

Sequencing procedure

The PTC-peptides were sequenced in a Beckman sequencer (Model 890 D) or in a new Knauer Model 810 modular liquid phase protein sequencer equipped on-line with an Knauer PTH (phenylthiohydantoin)-amino acid analyzer. PTH-amino acids were identified and quantified on an RP-HPLC system based on a C_{18} column (Knauer, West Berlin, Germany) and gradient elution with 6.5 m*M* sodium acetate–acetonitrile 1,2-dichloroethane (85:15:0.175) adjusted to pH 4.77 as buffer A and 100% acetonitrile as buffer B. Sequences were performed in the presence of polybrene using the wet-filter technique [9] in a new flow-through reactor [10].

RESULTS AND DISCUSSION

In peptide mapping, a protein is cleaved selectively by enzymes or by chemical digestion, and the resulting peptide mixture is separated by RP-HPLC to yield a peptide map or 'fingerprint'. This technique is one of the most useful applications of RP-HPLC since it provides information concerning expression errors, mutations, location of glycosylation, disulfide linkages, and structural identification of newly discovered or recombinant proteins, as well as being routinely used for the separation of peptides for sequencing analysis.

The sensitivity of detection of peptides in RP-HPLC is mainly limited by the transparency and purity of the mobile phase, and by the characteristic noise and drift of the detector [11]. In the case of analytical columns (3.9 mm I.D.), amounts of peptides in the range 0.5–1.0 nmol are routinely detected with a constant baseline for UV absorption at 220–230 nm, at sensitivities of approximately 2.0–0.05.

However, the ability to detect and manipulate peptides at the picomolar level in RP-HPLC requires the use of appropriate and very sensitive detection systems. A standard way to increase the sensitivity of detection on RP-HPLC is simply by using the scale expansion of the UV detector, but as can be seen in Fig. 1 (top) the increase in the baseline produced by the absorption of the organic solvent (acetonitrile) at 220 nm using three different sensitivities could limit the visualization of peptides in the chromatograms. To resolve the problem we investigated a new approach based on the pre-column derivatization of peptides using the Edman reagent PITC.

One of the advantages of using PITC is that in conventional water–acetonitrile–TFA elution systems in RP-HPLC the PTC derivatives can be detected with a flat, low baseline for UV absorption at 254–260 nm. As can be seen in Fig. 1 (bottom)

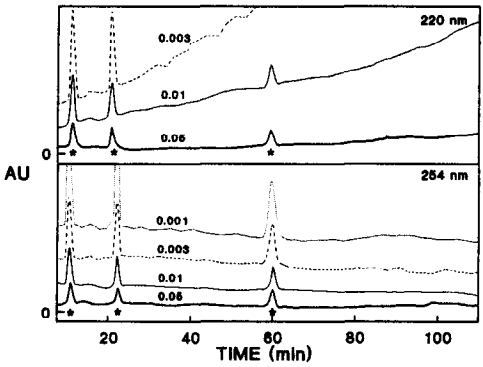

Fig. 1. Elution profile comparisons in the blank gradient produced by the absorption of the organic solvents during gradient elution at different wavelengths and sensitivities. Column: Nova-Pak (15 cm × 3.9 mm I.D.). The column was run at room temperature, at a flow-rate of 0.5 ml/min with a linear acetonitrile gradient from 20 to 40% containing 0.1% TFA. Sensitivities from 0.05 to 0.001 and absorbance at 220 nm and 254 nm were compared. The peaks corresponding to the derivatizing reagents are marked with an asterisk.

there is no increase in the baseline at 254 nm caused by the absorption of acetonitrile, even at a high sensitivity of detection, in contrast to the large increase in the baseline at 220–230 nm (Fig. 1, top).

Moreover, by using PITC the resulting PTC-peptides are in principle, suitable for Edman degradation, in contrast to other derivatizing reagents which have been described, such as fluorescamine [12], *o*-phthalaldehyde [13,14] or dansyl chloride [14], peptide derivatives of which are blocked at the N-terminal and consequently cannot be used for further sequence analysis.

Another reagent which has been used for pre-column derivatization of peptides at picomolar level, is dimethylaminoazobenzene isothiocyanate (DABITC). However, although the dimethylaminoazobenzene-thiocarbamoyl (DABTC)-peptides are also suitable for Edman degradation, DABITC has the disadvantage that its reaction with peptides is very slow, often incomplete, and several peptide derivatives are obtained from a single peptide [15].

In order to determine the separation and detection of PTC-peptides on RP-HPLC, aliquots of 5, 50 and 200 pmol of a tryptic digest of an L-chain immunoglobulin were derivatized with PITC and then injected into an RP-HPLC system as indicated in Experimental. The chromatographic distribution of the PTC peptide derivatives is shown in Fig. 2 and, as can be seen, a satisfactory resolution was obtained.

Fig. 2 also shows the typical effect of a direct UV detection of three different amounts of PTC-peptides (5, 50 and 200 pmol) monitored at 220 nm and three different scale expansions of the UV detector. Only in the chromatogram of 200 pmol are the PTC-peptides clearly detected either at 254 nm or with a slightly higher sensitivity at 220 nm (Fig. 2, left), while in the chromatogram of 5 and 50 pmol (Fig. 2, right), the increase in the baseline at high sensitivity does not permit the visualization of the PTC peptides at 220 nm and visualization can only be obtained at 254 nm (Fig. 2, right and inset). This technique is very sensitive since it permits the visualization of peptide maps at a level of 5 pmol at 260 nm with still acceptable

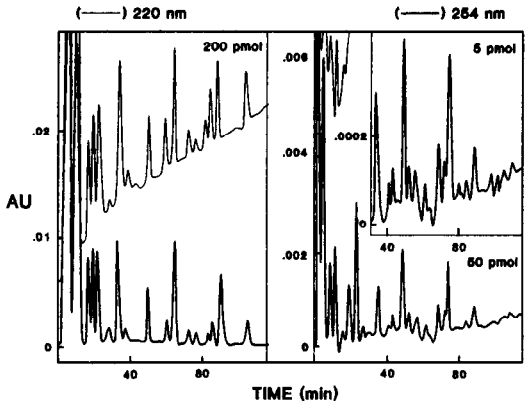

Fig. 2. Detection of different amounts of PTC-derivatized tryptic peptides at 220 and 254 nm at different sensitivities. Samples were 5, 50 or 200 pmol of a tryptic digest of an L-chain immunoglobulin derivatized with PTC. Peptides were eluted at room temperature on a gradient of acetonitrile containing 0.1% TFA as indicated in Fig. 1. The corresponding PTC-peptides identified by amino acid analysis in each chromatogram are numbered. The peaks corresponding to the derivatizing reagents are marked with an asterisk.

baseline using a high sensitivity of detection (Fig. 2). These data demonstrate that the PTC-peptides can be adequately separated at the picomolar level by RP-HPLC.

The elution pattern of PTC-peptides in RP-HPLC (Fig. 2) can be seen to be satisfactory but different from that obtained with underivatized peptides. In general we observed that PTC-peptides in RP-HPLC are eluted at a higher concentration of acetonitrile than underivatized peptides, under the same chromatographic conditions (data not shown).

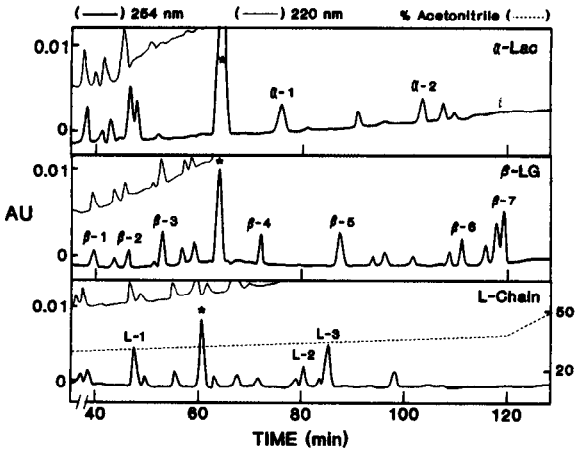

Fig. 3. Fractionation by RP-HPLC of PTC-derivatized tryptic peptides (300 pmol) from α-lactalbumin (α-Lac), β-lactoglobulin (β-LG) and a light-chain (L-chain) immunoglobulin. Peptides were eluted at room temperature on an acetonitrile gradient containing 0.1% TFA as indicated in the figure. Flow-rate: 0.5 ml/min. The peaks corresponding to the derivatizing reagents are marked with an asterisk. All peaks in the chromatograms are PTC-peptides which were identified by amino acid analysis. Only those PTC-peptides which were used for sequence studies have been labelled.

The possibility of subjecting the PTC-peptides isolated from RP-HPLC to Edman degradation analysis was also investigated.

For this purpose aliquots containing 300 pmol of the peptide derivatives of the proteins α-lactalbumin, β-lactoglobulin and L-chain were analyzed by RP-HPLC, and the chromatographic distribution of the corresponding PTC-peptides is shown in Fig. 3. The calculated average yield of the isolated PTC-peptides in the three chromatograms as determined by amino acids analysis, was approximately 80–90% of the applied material. The amino acid composition data indicate that a single PTC derivative of each peptide is obtained, while DABITC gives several derivatives [15].

Aliquots of 250 pmol of the PTC-peptides indicated by numbers in the three peptide maps of Fig. 3 were either subjected to automatic degradation or maintained for 1–30 days in the freezer before being used for sequence analysis. The results of the amino acid sequences obtained from these PTC-peptides are shown in Table I. This also demonstrates that the PTC-peptides are suitable for sequencing analysis and that they are stable for a relatively long period of time if stored frozen (−20°C).

It is noteworthy that in general the yield of the first cycle of these PTC-peptides was always low (Table I) (in comparison with the initial yield of non-derivatized

TABLE I

AMINO ACID SEQUENCE OF TRYPTIC PTC-PEPTIDES FROM α-LACTALBUMIN, β-LACTOGLOBULIN AND L-CHAIN

Residue numbers in parenthesis refer to primary sequence position in α-lactalbumin[16], β-lactoglobulin [17] and (ESC) L-chain (unpublished results). The yield (pmol) of the two PTH-amino acids obtained in the first cycle are indicated below the sequences.

PTC-peptide	Amino acid sequence	Position
α-1	Glu–Gln–Leu–Thr–Lys (22,68)	(1–5)
α-2	Ile–Leu–Asp–Lys (75,225)	(95–98)
β-1	Lys–Ile–Pro–Ala–Val–Phe (99,114)	(77–82)
β-2	Leu–Ser–Phe–Asn–Pro–Thr–Gln–Leu (142,60)	(149–156)
β-3	Tyr–Leu–Leu–Phe–Cys–Met (62,47)	(102–107)
β-4	Leu–Ile–Val–Thr–Gln–Thr–Met–Lys (36,49)	(1–8)
β-5	Ser–Leu–Ala–Met–Ala–Ala–Ser–Asp–Ile–Ser (25,35)	(21–30)
β-6	Thr–Lys–Ile–Pro–Ala–Val (12,33)	(76–81)
β-7	Lys–Val–Ala–Gly–Thr–Trp–Tyr–Ser–Leu–Ala (30,188)	(14–23)
L-1	Tyr–Ala–Ala–Ser–Ser–Tyr–Leu–Ser–Leu–Thr–Pro (15,109)	(173–183)
L-2	Phe-Ser-Gly-Ser-Lys (45,151)	(62–66)
L-3	Ala–Gly–Val–Glu–Thr–Thr–Lys–Pro–Ser–Lys (112,245)	(57–166)

peptides) regularly obtained in our sequencer, however, the yield in the second and subsequent cycles was normal. This is probably because the PTC-amino acid of the first residue undergoes several manipulations, such as manual derivatization, HPLC fractionation and peak collection, which are not done routinely in automatic degradation of non-derivatized peptides. The PTC-peptides can be sequenced using either a regular Edman degradation or a modified one, in which, in the coupling step, the PITC is eliminated from the first cycle, since the PTC-peptide is already formed. In both cases, a single PTH-amino acid is obtained.

A peculiarity observed during the sequencing of PTC-peptides using our wet-filter sequencer was that two different PTH derivatives were obtained in the first cycle, corresponding to the first and second amino acids. This is because, with the program used, prior to initiating the first cycle the PTC-peptide (or sample), containing membrane is gently treated with 100% TFA, and under this acid condition the first PTC-amino acid is liberated from the PTC-peptide. Then, during the coupling step in this first cycle, the PITC reacts with the nascent amino group from the second residue of the peptide, and consequently at the end of this cycle two PTH-amino acids are obtained. Again the yield of the PTH corresponding to the first amino acid is always lower (30–55%) than that of the second amino acid (50–70%) (data not shown). The possibility that the second signal for the first amino acid may be due to the presence of TFA in the RP-HPLC cannot be discounted.

In conclusion, we have shown that the pre-column PITC derivatization procedure described is a simple general procedure which can be used as a very sensitive peptide-mapping method at the picomolar level (5–10 pmol).

As stressed above, in comparison with other reagents, including DABITC, the advantage of using PITC is that the reaction with peptides is complete, reproducible and the peptide derivatives are stable for weeks. In addition the PTC-peptides can be properly fractionated and purified by RP-HPLC with a high degree of resolution. The recovery of the PTC-peptides isolated appears to be quantitative, and the PTC-peptides are suitable for microsequence analysis.

One of the disadvantages of this precolumn derivatization system using PITC is the presence in the chromatograms of several artefacts (Figs. 1–3), resulting from by products of the reagents which can coelute with some PTC-peptides. Several extraction procedures to remove these byproducts have been tried, with unsuccessful results (data not shown).

Nevertheless, this precolumn derivatization procedure can be used as a valid alternative to generate peptide maps at the picomolar level to obtain internal sequence information (either of proteins or of blocked proteins of which the direct primary structure cannot be determined by automatic sequencing) for gene identification or to generate oligonucleotides which can be used to clone genes.

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