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Identification of peptides containing aromatic amino acids, cysteine, iodotyrosine and iodothyronine by high-performance liquid chromatography with photodiode-array detection

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ABSTRACT

A general method for the identification of peptides containing tyrosine, tryptophan, phenylalanine and cysteine and a preliminary study of the identification of peptides containing monoiodotyrosine, diiodotyrosine, triiodothyronine and thyroxine by high-performance liquid chromatography photodiode-array ultraviolet–visible detection is reported. The technique was tested with an immunoglobulin light chain and with an *in vitro* iodinated urinary human complex-forming glycoprotein, heterogeneous in charge (protein HC), and human thyroglobulin (Tg) after enzymatic digestion. The system continuously monitors wavelengths and collects data that can be analysed by comparison with standard spectra via software routines. This procedure saves sample, time and reagents and avoids the use of radioactive reagents.

INTRODUCTION

The detection of peptides containing specific amino acids or their derivatives is of importance in protein studies in order to proceed to their characterization. Since the introduction of high-performance liquid chromatography (HPLC), many different procedures for the identification of peptides containing aromatic amino acids[1,2] and cysteine[3] have been described. The separation by HPLC of peptides containing monoiodotyrosine (MIT), diiodotyrosine (DIT) and thyroxine (T_4) from *in vivo* iodinated, non-radiactive thyroglobulin (Tg) has been reported[4]; a multi-wavelength detector was used, recording the absorbance at 230 nm to detect peptides, at 325 nm to detect sites of iodination [MIT, DIT, triiodothyronine (T_3) and T_4] and at 350 to detect T_4. More recently, a method for the separation, detection, hydrolysis and storage of

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iodine and fluorine derivatives of thyrosine peptides by reversed-phase (RP) chromatography at various pH levels[5] and a method for the identification of iodoamino acids in peptide sequences of Tg, iodinated *in vitro* with radioiodine[6], has been described. However, none of them is simple and they require a series of separation steps prior to detection.

In the last few years, photodiode-array (PDA) ultraviolet (UV) or ultraviolet–visible (UV–VIS) detectors have been employed as components of HPLC systems. The advantage of these detectors is that they can continuously monitor all wavelengths of the spectrum and collect the data for future processing. After chromatography the data can be analysed via software routines, such as spectral analysis for peak identification and purity confirmation or comparison of absorption spectra of separated peaks, to obtain specific information about the sample. Recently, metabolites of aromatic amino acids[7], mycotoxins and other fungal metabolites[8], protein HC[9] and several chromophores[10,11] have been analysed with this detection system. This study was focused on peptide characterization and a PDA detector was used for identification of peptides containing aromatic amino acids, cysteine, MIT, DIT, T_3 and T_4.

In this paper we report a general procedure that allows the identification of all the above-mentioned peptides, using several simple data processing modes: "spectrum analysis", "second-derivative spectra", "spectrum index plot" and "multichromatogram analysis".

EXPERIMENTAL

Acetonitrile was obtained from Scharlau (Barcelona, Spain), guanidinium chloride, dithiothreitol, pronase, lactoperoxidase, glucose oxidase, MIT, DIT, T_3 and T_4 from Sigma (St. Louis, MO, U.S.A.), 4-vinylpyridine from Aldrich (Milwaukee, WI, U.S.A.) and tetrahydrofuran, 1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, trifluoroacetic acid (TFA) and other compounds from Merck (Darmstadt, F.R.G.). Ultrapure water for HPLC was generated by a Milli-RO4, coupled to a Milli-Q water-purification system (Millipore, Bedford, MA, U.S.A.) and was used in the preparation of all buffers.

The chromatograph consisted of two Waters Assoc. (Milford, MA, U.S.A.) M6000A pumps, a Waters Assoc. 680 automated gradient controller and a Waters Assoc. 990 PDA detector with a dynamic range from the ultraviolet to the visible region (190–800 nm), based on an NEC APC III personal computer. All sample injections were performed with a Waters Assoc. U6K universal injector.

Size-exclusion HPLC was performed on a TSK 3000 SWG column (300 × 21.5 mm I.D.) (Toyo Soda, Tokyo, Japan), fitted with a TSK 3000 SWG guard column, by isocratic elution with 0.1 *M* ammonium acetate buffer (pH 5.0). The column was operated at room temperature at flow-rate of 1 ml/min.

RP-HPLC was performed with a NovaPak column (150 × 3.9 mm I.D.) (Waters Assoc.), protected by a guard column packed with μBondapak C_{18}/Corasil (Waters Assoc.). The column was eluted with acetonitrile gradients containing 0.1% (v/v) TFA (pH 2.0). The TFA-insoluble material was solubilized with 6 *M* guanidinium chloride and eluted with acetonitrile gradients containing 0.7% (w/v) ammonium hydrogencarbonate (pH 8.0). The column was operated at room temperature at a flow-rate of 0.5 ml/min.

The urinary protein HC preparation was provided by Dr. A. O. Grubb (Lund University, Sweden). Protein HC was purified as described earlier[12]. A completely reduced and S-pyridylethylated λ-light chain, isolated from a human monoclonal IgM (ESC), was used for this study.

Iodine-poor (0.01% I) human Tg from a multinodular goitre kindly provided by Dr. Geraldo Medeiros Neto (Laboratorio de Tiroides, Hospital das Clinicas, Facultad de Medicina de Sao Paolo, Brazil) was purified as described previously[13].

Urinary protein HC and Tg were iodinated enzymatically *in vitro* in 0.067 *M* phosphate buffer (pH 7.0) at 37°C with 10^{-4} *M* iodide, labelled with radioiodine (I*, specific activity 1.0 μCi/μat), 1.5 μg/ml lactoperoxidase, 1.0 mg/ml glucose and 1.5 μg/ml glucose oxidase for 1 h and 15 min, respectively. The number of atoms of iodine bound per mole of protein was calculated as described previously[13].

A 3-mg amount of human monoclonal IgM, dissolved in 200 μl of 2 *M* Tris–HCl buffer (pH 8.6) containing 0.002 *M* EDTA and 6 *M* guanidinium chloride, was incubated with 35 m*M* dithiothreitol for 120 min at 37°C. S-Pyridylethylation was achieved by adding 5.0 μl of 4-vinylpyridine and incubating for 15 min at room temperature. The excess of reagents was removed by HPLC on a TSK-3000 SWG column (300 × 21.5 mm I.D.) in 0.1 *M* ammonium acetate buffer (pH 5.0).

Reduced and S-pyridylethylated λ-light-chain immunoglobulin (115 μg) was digested with 1.15 μg of TPCK-trypsin in 250 μl of 0.2 *M* N-methylmorpholine acetate buffer (pH 8.2) for 2 h at 37°C. After digestion, the material was freeze-dried, lyophilized and redissolved immediately before chromatographic analysis in 0.1% (v/v) TFA.

The iodinated Tg was reduced with mercaptoethanol (ME) (100 mol ME/mol S–S in Tg) and subsequently S-cyanoethylated with acrylonitrile (2 mol/mol ME added), stopping the reaction with ME (2 mol/mol acrylonitrile added). The excess of reagents was eliminated by passage through an Econopac 10DG disposable chromatographic column (Bio-Rad Labs., Richmond, CA, U.S.A.). Tg fractions were pooled and digested with TPCK-trypsin (5%, w/w) for 16 h at 37°C.

Aliquots from iodinated protein HC and Tg were digested with pronase and the iodoamino acid distribution was determined as described previously[13].

MIT, DIT, T_3 and T_4 standards were dissolved in 50% aqueous acetonitrile containing 0.1% (v/v) TFA or in 0.7% aqueous ammonium hydrogencarbonate containing 5% acetonitrile, injected into a NovaPak C_{18} column, and eluted with 50% aqueous acetonitrile containing 0.1% (v/v) TFA or with 52.5% aqueous acetonitrile containing 0.35% (w/v) ammonium hydrogencarbonate. The absorption spectrum was obtained using a Waters Assoc. 990 PDA detector.

RESULTS AND DISCUSSION

The conventional way to identify aromatic Tyr- and Phe-containing peptides in HPLC is by acid hydrolysis, followed by amino acid analysis. However, this procedure cannot be used for Trp-containing peptides, as Trp is partly destroyed by the action of the acid, and consequently an additional technique to identify them must be used[14]. The above-described procedures require the use of additional sample- and time-consuming detection techniques. Alternatively, in HPLC the aromatic amino acid-containing peptides can be also localized simply by monitoring the chromatogram at

260 or 280 nm. However, this system itself does not distinguish precisely among the three aromatic amino acid-containing peptides, and consequently further analysis must be employed.

To solve this problem, we have used a routine procedure with a PDA detector adapted to our HPLC system in order to identify immediately after separation each of the Tyr-, Trp- and Phe-containing peptides. Fig. 1A shows a typical chromatogram, monitored at 220 nm by PDA detection and corresponding to a tryptic digest of a λ-light-chain immunoglobulin, chromatographed on an RP-HPLC column. By using the "spectrum index plot" data program, it is possible to obtain automatically the absorption spectrum at any wavelength range in the peak maximum from each peak of the chromatogram (Fig. 1B). By selecting the spectra from 240 to 310 nm, it is possible to recognize unequivocally by inspection of these spectra the three aromatic amino acid-containing peptides. A typical Phe spectrum with a maximum at 254 nm can be observed in peak 1, which corresponds to a Phe-containing peptide. Tyr-containing peptides can also be immediately recognized by inspection of the characteristic spectrum with a maximum around 278 nm and returning to the baseline at around 290 nm in peaks 6, 7, 12, 13, 14 and 15. Trp-containing peptides can be identified in peaks 8, 9, 10, 11, 16 and 17 by their spectrum with a maximum at 280 nm but not returning to baseline until 310 nm.

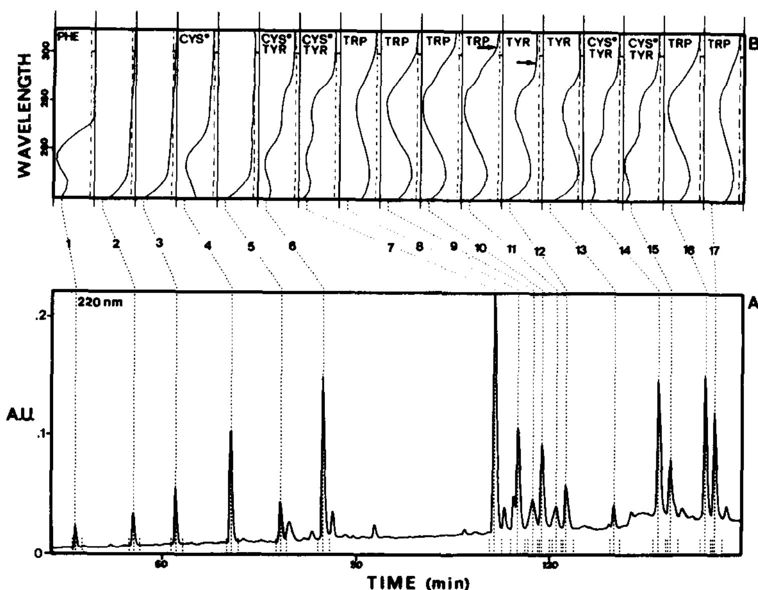

Fig. 1. Separation of a tryptic digest of reduced and S-pyridylethylated λ-light-chain immunoglobulin (ESC). Sample, 5.0 nmol; column, NovaPak C_{18} (300 × 21.5 mm I.D.); flow-rate, 0.5 ml/min. The column was equilibrated with 0.1% (v/v) aqueous TFA, and peptides were eluted at room temperature, using a linear gradient from 0 to 80% of acetonitrile containing 0.1% (v/v) TFA. (A) The chromatogram was analysed by monitoring the absorbance at 220 nm. (B) Automatic spectra were acquired from the peak maxima from 240 to 330 nm. Arrows indicate the end of the spectrum of Trp and Tyr. CYS*, TRP, TYR and PHE correspond to peptides containing S-4PE-cysteine, triptophan, tyrosine and phenylalanine, respectively.

The presence of Phe and Tyr in these peaks was verified by amino acid analysis and Trp was identified by sequence studies (data not shown). The additional use of the program "spectrum analysis" provides a useful help in the comparison of the spectra of the Phe-, Tyr- and Trp-containing peaks and with standard aromatic amino acids, as shown in Fig. 2, which displays the normalized spectra corresponding to peaks 1, 6, 12 and 10 in Fig. 1.

Another amino acid generally considered to be difficult to identify during peptide purification is cysteine. In HPLC, Cys-containing peptides can be identified either by the presence of cysteic acid or carboxymethylcysteine in the amino acid composition of their hydrolysates or by the radioactivity of labelled carboxymethylcysteine-containing peptides. These procedures require the use of an amino acid analyser or scintillation counter, which are both time and sample consuming. More recently, Cys in peptides has been identified as 4-pyridylethylcysteine (4-PE-Cys) from S-pyridylethylated proteins[3]. Based on the fact that this reagent has an extremely high molar absorptivity at 254 nm compared with the three aromatic amino acids, 4-PE-Cys-containing peptides can be easily localized in a HPLC trace by monitoring the absorbance at 254 nm. Fig. 3 shows in the "multichromatogram analysis" program the two patterns at 254 and 220 nm, corresponding to the tryptic digest of reduced and S-pyridylethylated λ-light chains of IgM (Fig. 1). Visual inspection of the chromatogram at 254 nm shows the presence of four major and a few minor peaks. Characterization of the 4-PE-Cys-containing peptides was possible by means of the program "spectrum index" without recourse to amino acid analysis (Fig. 1). As can be seen in Fig. 1B, the spectra corresponding to peaks 4, 6, 7, 14 and 15 shows a characteristic maximum at 254 nm, indicating the presence of 4-PE-Cys-containing peptides. This system also allows the identification of the minor peaks, 16 and 17, detected at 254 nm as Trp-containing peptides instead of 4-PE-Cys derivatives. In both Figs. 1B and 2 these programs allow the easy identification of peptides containing more than one chromophore, such as Cys and Tyr in peaks 6, 7, 14 and 15.

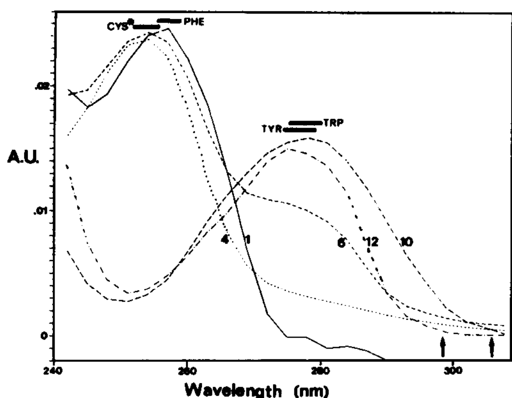

Fig. 2. Comparative spectral analysis from 240 to 310 nm of peaks 1, 4, 6 and 12 from Fig. 1. Spectra were normalized in order to eliminate concentration differences. Arrows show the end of the absorption spectrum of tryptophan and tyrosine. Horizontal bars represent the range around the maxima for S-4PE-Cys, Trp, Tyr and Phe standards.

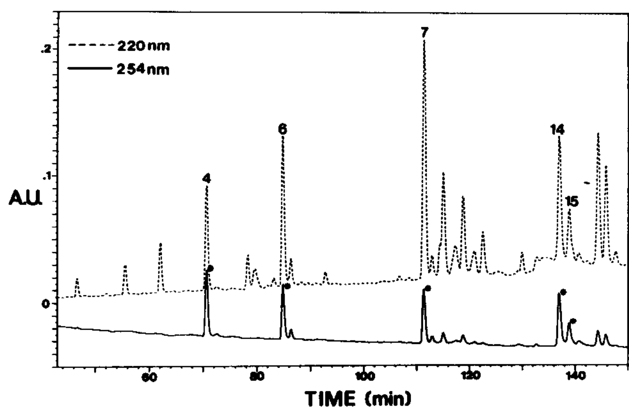

Fig. 3. Multi-chromatographic analysis of the absorbance at 220 and 254 nm of tryptic peptides from Fig. 1. The peaks marked with asterisks represent S-4PE-Cys-containing peptides. The peak numbers correspond to peaks in Fig. 1.

We investigated the direct ditection of iodoamino acid-containing peptides, both in Tg (which is the Tyr-containing protein forming thyroid hormones with the highest efficiency by iodination and coupling of some of its Tyr residues) and in protein HC, which does not form thyroid hormones efficiently after *in vitro* iodination. As a preliminary step to the detection of MIT-, DIT-, T_3- and T_4-containing peptides in both protein HC and Tg, standards were injected into the HPLC system in both TFA (pH 2.0) and ammonium hydrogencarbonate (pH 8.0). Using the "spectrum analysis" program (Fig. 4A), the absorbance maxima in TFA for MIT, DIT, T_3 and T_4 were 284, 290, 298 and 302 nm, respectively. These data agree with those reported earlier[5] and show a gradual shift towards higher wavelengths with increasing number of iodine atoms in the molecule. Although the spectra of T_3 and T_4 have similar maxima around 300 nm, the spectrum of T_3 ends at about 334 nm whereas that of T_4 ends at about 340 nm. The "second-derivative" program (Fig. 4B) shows that for each iodoamino acid there is a characteristic maximum around 304, 310, 320 and 330 nm for MIT, DIT, T_3 and T_4, respectively. The clearly different absorption and second-derivative maxima of each iodoamino acid allow their identification.

Fig. 5 shows the 220 nm and the ^{125}I radioactivity patterns of a partial hydrolysate of *in vitro* iodinated protein HC, chromatographed on an RP-HPLC column. Peaks 47 and 87 contained 85.5% and 92.5% MIT, respectively, whereas peaks 93 and 273 contained 90.0% and 82.2% DIT, respectively, as shown by pronase digestion and paper chromatography (Table I). This was confirmed by using the "spectral analysis" and "second-derivative" programs, as shown in Fig. 6A and B, respectively, as the spectrum of fraction 87 coincided exactly with that of the MIT standard, and the spectra of fractions 93 and 273 coincided with that of the DIT standard. However, peak 47 does not coincide completely with either the MIT or the DIT standard by spectral analysis (Fig. 6A), probably owing to the contribution of aromatic amino acid contaminants, as the absorbance peak and the ^{125}I peak around that fraction do not coincide (Fig. 5). However, when using the "second-derivative"

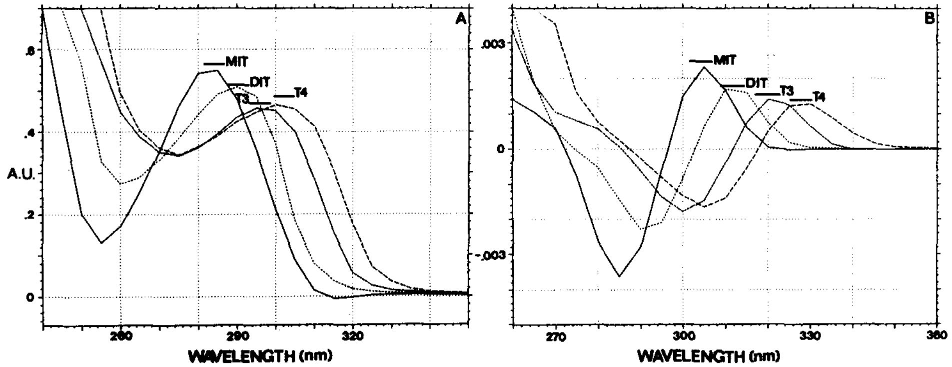

Fig. 4. Comparative spectral analysis from 240 to 350 nm (A) and second-derivative spectra from 260 to 360 nm (B) of MIT, DIT, T_3 and T_4 standards. Horizontal bars represent the range around the spectra maximum. Spectra were normalized in order to eliminate concentration differences.

program, the maximum and the minimum of fraction 47 are closer to those of MIT, strongly suggesting that the main iodoamino acid present is MIT.

Fig. 7A shows the absorption spectra of MIT, DIT, T_3 and T_4 standards and that of a T_4-rich peptide obtained by tryptic digestion of an *in vitro* iodinated Tg containing 23.4 atoms of iodine per mole of protein and 11.7% T_4. After pronase digestion of the fractions, it was seen that peak 137 contained as much as 77.0% T_4 (Table I). Although its maximum was at 285 nm, not coinciding with that of T_4, it absorbed beyond 340 nm as only the T_4 standard does. When using the "second-derivative" program (Fig. 7B), the maximum was closer to that of the DIT standard, but again it absorbed beyond 340 nm. Hence the most specific criterion to detect

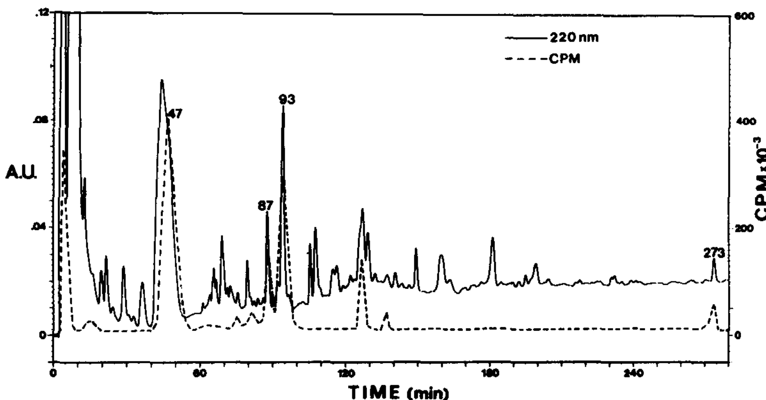

Fig. 5. Fractionation of a pronase digest of urinary ^{125}I-labelled protein HC. Sample, 31.0 nmol; column, NovaPak C_{18} (300 × 21.5 mm I.D.); flow-rate, 0.5 ml/min. The column was equilibrated with 0.1% (v/v) aqueous TFA, and peptides were eluted at room temperature, using a linear gradient from 0 to 80% of acetonitrile containing 0.1% (v/v) TFA. Fractions of 2 ml were collected and the radioactivity was measured.

TABLE I

IODINE ATOMS BOUND AND IODOAMINO ACID DISTRIBUTION OF *IN VITRO* IODINATED PROTEIN HC, THYROGLOBULIN AND PEPTIDES FROM TRYPTIC HYDROLYSIS

Species	*I (atoms/mol)*	*Labelled iodoamino acid distribution (%)*			
		DIT	*MIT*	T_4	T_3
Protein HC	2.0	14.2	62.7	0.8	0
Peptide 47		1.8	85.5	0.3	0.5
87		1.6	92.5	0.4	0.2
93		90.0	3.2	0.8	0.4
273		82.2	4.3	0.6	0.25
Thyroglobulin	23.4	43.5	36.2	11.7	1.5
Peptide 137		6.9	3.5	77.0	1.5

a T_4-containing peptide is the detection of significant absorbance beyond 340 nm. The fact that this T_4-rich peptide has a maximum absorption around 285 nm and a second-derivative maximum around 312 nm could be due to a high aromatic amino acid content. Both the "spectrum analysis" and the "second-derivative" programs seem to be complementary and are helpful in the direct detection of iodotyrosine- and iodothyronine-containing peptides.

In conclusion, we have described a procedure that permits the identification by HPLC of aromatic amino acids (Tyr, Trp and Phe) and cysteine (4-PE-Cys) using a PDA detector. In addition, a prelminary report on the direct detection of iodotyronine (MIT, DIT)- and iodothyronine (T_3, T_4)-containing peptides has been given. The direct detection of these peptides, even when they are still impure, is very useful for the identification and follow-up of the selected peptides during their HPLC purification. In addition to avoiding the need for radioactive compounds, this system permits the identification of peptides immediately after HPLC with corresponding savings of sample, time and reagents.

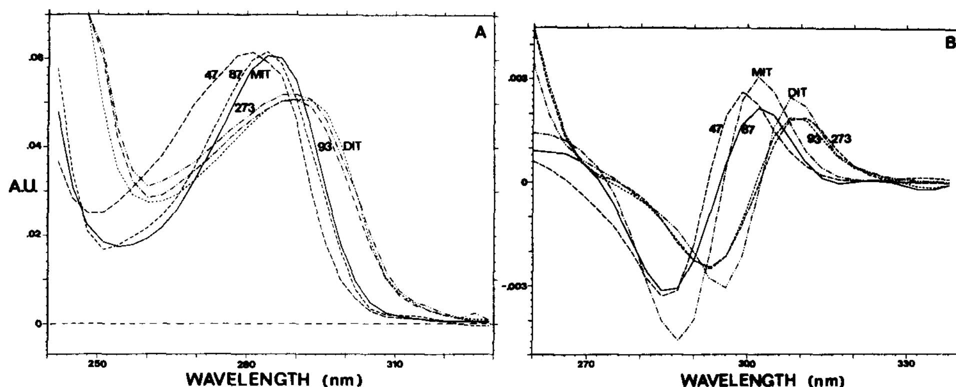

Fig. 6. Comparative spectral analysis from 240 to 330 nm (A) and second-derivative spectra from 240 to 340 nm (B) of protein HC pronase peptides 47, 87, 93 and 273 from Fig. 5. The spectra of MIT and DIT are included for comparison. Spectra were normalized in order to eliminate concentration differences.

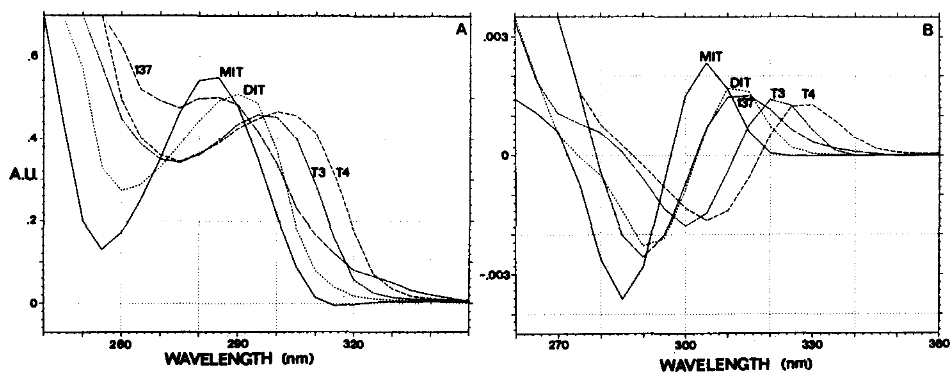

Fig. 7. Comparative spectral analysis from 240 to 350 nm (A) and second-derivative spectra from 260 to 360 nm (B) of a T_4-rich tryptic peptide (137) from *in vitro* iodinated thyroglobulin. The spectra of MIT, DIT, T_3 and T_4 are included for comparison. Spectra were normalized in order to eliminate concentration differences.

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