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USE OF SCANNING DIODE ARRAY DETECTOR WITH REVERSED-PHASE MICROBORE COLUMNS FOR THE REAL-TIME SPECTRAL ANALYSIS OF AROMATIC AMINO ACIDS IN PEPTIDES AND PROTEINS AT THE SUB-MICROGRAM LEVEL

APPLICATIONS TO PEPTIDE AND PROTEIN MICROSEQUENCING

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SUMMARY

The characteristic zero- and second-order derivative spectra of phenylalanine, tyrosine and tryptophan are described and used to identify aromatic residues contained in sub-microgram amounts of polypeptides and proteins during their elution from reversed-phase short microbore columns under gradient conditions. Spectral data were acquired with a commercially available scanning diode array detector. The method allows the non-destructive identification of tryptophan residues in complex polypeptide mixtures such as tryptic maps and enables the selection and isolation of such peptides for amino acid sequence analysis. The sub-microgram level of sensitivity is due to the small peak volumes and consequent elevated solute concentrations obtained on short (< 10 cm), microbore (2 mm I.D.) reversed-phase columns.

INTRODUCTION

The recent introduction of rapid-scanning UV–VIS detectors based on linear arrays of diodes provides the biochemist with a powerful analytical tool when used in conjunction with high-performance liquid chromatography (HPLC) methodologies. A major advantage of multi-channel detectors is that spectra of eluting compounds can be stored in a digital form by bench-top computers and then manipulated by a variety of algorithms to ascertain the purity of an eluting peak[1–3]. Such spectral data, in combination with monitoring of the eluate at chosen discrete wave-

lengths, greatly increases detection specificity over that available from single channel detectors.

Peptides and proteins exhibit UV-absorption spectra characteristic of their component amino acids[4–6], particularly the aromatic residues phenylalanine, tyrosine and tryptophan. However, analysis of peptides and proteins for the detection of constituent aromatic residues by UV-absorption spectroscopy poses problems because of overlapping absorption bands. Derivative spectroscopy enhances resolution of minor spectral features characteristic of specific chromophores[7], thus overcoming some of the problems of interference in the UV-absorption spectrum. Derivative spectroscopy has been widely used for the analysis of aromatic amino acids contained in native and denatured proteins[8–16], under non-chromatographic conditions. In particular, the second-order derivative, which transforms peaks and shoulders into minima, has gained the widest use[12–16]. The aim of this study was to investigate the use of zero- and second-order derivative spectroscopy for the identification of aromatic residues contained in polypeptides and proteins during their elution under gradient conditions from reversed-phase HPLC short microbore columns. We have developed the use of reversed-phase short microbore columns for the purification and concentration of sub-microgram amounts of polypeptides and proteins in a form suitable for gas-phase sequence analysis[17–20]. Thus, combining the enhanced sensitivity afforded by microbore columns[17] with the high-speed scanning ability of diode array detectors it is possible to obtain UV spectral data on eluting peptides and proteins at the sub-microgram level.

MATERIALS AND METHODS

Chemicals and reagents

HPLC-grade organic solvents were purchased from Mallinckrodt (Melbourne, Australia). Ammonium bicarbonate, trifluoroacetic acid (TFA) and all other chemicals used were AnalaR grade or better from BDH (Poole, U.K.). All HPLC solvents were prepared with water purified by a tandem MilliRo–MilliQ system from Millipore (Bedford, MA, U.S.A.). Peptides numbered 1–22 in Table I were purchased from Sigma (St. Louis, MO, U.S.A.). The isolation and sequence determination of tryptic peptides of murine transferrin receptor[18,21] and murine plasma cell antigen PC-1[19,22] have been reported. Murine and rat epidermal growth factor (EGF) were purified by HPLC as reported[23,24]. Porcine transferrin was purified from gastric mucosa and serum and partial sequence determined[25]. The isolation, partial amino acid sequence and DNA sequence of murine granulocyte macrophage colony stimulating factor (GM-CSF) have been reported[26–28]. All other proteins in Table I were purchased from Sigma.

Equipment

All peptides and proteins were chromatographed on a Hewlett-Packard liquid chromatograph Model 1090 equipped with a Model 1040A diode array detector. Spectral and chromatographic data were stored on disc using a Hewlett-Packard Model 85B computer and a Model 9121 twin disc drive. Zero- and second-order spectra were obtained with the EVALU 1 and 2 software packages obtained from Hewlett-Packard (Waldbronn, F.R.G).

Methods

Peptides and proteins at the sub-microgram level were chromatographed on the following short microbore columns: (i) Hypersil C_8 (5 μm dimethyloctylsilica, 12 nm pore size, packed into a 100 × 2.0 mm I.D. column) obtained from Hewlett-Packard; (ii) Vydac C_4 (5 μm dimethylbutylsilica, 30 nm pore size, packed into a 75 × 2 mm I.D. column) which was obtained from The Separations Group, CA, U.S.A., and packed in our laboratory[18]; (iii) Brownlee C_8 (10 μm dimethyloctylsilica, 30 nm probe size, packed into a 30 × 2.0 mm I.D. column) obtained from Brownlee Labs., Santa Clara, CA, U.S.A. Columns were developed with linear 30- or 60-min gradients in acetonitrile using either trifluoroacetic acid (pH 2.0) or ammonium bicarbonate (pH 7.8) as the mobile phase ionic components, as described previously[18]. Flow-rates were 100 μl/min and the column temperatures were maintained at 45°C for polypeptides and 40°C for the proteins. At the completion of each chromatographic run zero- and second-order derivative spectra were plotted and aromatic residues identified by their characteristic second-derivative minima.

RESULTS AND DISCUSSION

Spectral analysis of model polypeptides

Zero- and second-order derivative spectra were initially obtained for sub-microgram amounts of model polypeptides (1–22 in Table I) of defined aromatic amino acid composition during gradient elution from reversed-phase short microbore columns. The resultant derivative spectra were critically examined for characteristic bands of each aromatic amino acid and used in the identification of the constituent aromatic residues in polypeptides and proteins of unknown composition under similar sample levels and chromatographic conditions. Zero- and second-order derivative spectra characteristic of phenylalanine, tyrosine and tryptophan are shown in Fig. 1a–c. The observed characteristic minima in the second-order derivative spectra of phenylalanine at 257 ± 2 nm, of tyrosine at 280 ± 2 nm, and of tryptophan at 290 ± 2 nm, are in accord with published data obtained from spectral studies on these aromatic amino acids[10–16] but performed under non-chromatographic conditions.

In addition to the characteristic second-order minima described above, additional minima of lower intensity were observed for each aromatic amino acid. Thus, for phenylalanine (Fig. 1a) two local minima could be identified, at 250 ± 2 nm and 264 ± 2 nm. Similarly, two local minima, at 268 ± 2 nm and 278 ± 2 nm, were observed for tryptophan (Fig. 1c). In the case of tyrosine there was one local minimum, at 272 ± 2 nm (Fig. 1b). Since there was some overlap in these minima, zero- and second-order spectra of dipeptides of mixed aromatic amino acid composition were examined in order to examine the specificity of spectral identification of aromatic residues. The zero- and second-order spectra of the dipeptide Trp–Phe (Fig. 1d), demonstrate that the characteristic second-order minimum for tryptophan at 290 ± 2 nm is clearly much stronger than the minimum at 257 ± 2 nm characteristic of phenylalanine. However, by decreasing the attenuation of the second-order derivative plot by a factor of twenty, it is possible to discern the spectral contribution of phenylalanine at 257 ± 2 nm, on a sloping background (Fig. 1e). The identification of tyrosine in the presence of tryptophan was ambiguous when the tyrosine-to-tryptophan ratio was 1:1, as demonstrated by the dipeptide Trp–Tyr (Fig. 1e). This am-

TABLE I

REAL-TIME SPECTRAL ANALYSIS OF DEFINED AMINO ACIDS, PEPTIDES AND PROTEINS DURING ELUTION FROM MICROBORE COLUMNS

The single letter code is used for the amino acids[31]. Sample amounts for polypeptides 1–19, 23, 26, 28, 29 and 31 were obtained by dilution of stock solutions which had been prepared by dissolving a weighed amount of protein into the running buffer. For the other polypeptides and proteins, sample amount was determined by amino acid analysis. Zero- and second-order derivative spectroscopy was used to identify aromatic amino acids contained in each solute. The symbol p denotes presence and the symbol a denotes absence of a particular aromatic residue. The success of spectral analysis in correctly identifying aromatic residues is denoted by + and − which signify a non-ambiguous or ambiguous determination, respectively.

Compound	*Composition*	*Amount*	*Identification of aromatic residues*		
			F	*Y*	*W*
1 F	(F)	0.05	p+	a+	a+
2 FF	(F)	0.05	p+	a+	a+
3 FFF	(F)	0.05	p+	a+	a+
4 FFFF	(F)	0.08	p+	a+	a+
5 FFFFF	(F)	0.08	p+	a+	a+
6 Y	(Y)	0.05	a+	p+	a+
7 YY	(Y)	0.05	a+	p+	a+
8 YYY	(Y)	0.05	a+	p+	a+
9 W	(W)	0.05	a+	a−	p+
10 WW	(W)	0.05	a+	a−	p+
11 WF	(F, W)	0.05	p+	a−	p+
12 WY	(Y, W)	0.05	a+	p−	p+
13 AW	(W)	0.05	a+	a−	p+
14 YA	(Y)	0.05	a+	p+	a+
15 FY	(F, Y)	0.05	p+	p+	a+
16 AA		0.05	a+	a+	a+
17 YVMGHFRWDRFG	(2F, Y, W)	0.12	p+	p−	p+
18 YGGFM	(F, Y)	0.06	p+	p+	a+
19 YGGFL	(F, Y)	0.06	p+	p+	a+
20 DRVYIHPFHL	(F, Y)	0.08	p+	p+	a+
21 DRVYIHPF	(F, Y)	0.08	p+	p+	a+
22 RVYIHPE	(F, Y)	0.06	p+	p+	a+
23 b insulin	(3F, 4Y)	0.3	p+	p+	a+
24 mEGF	(5Y, 2W)	0.5	a+	p+	p+
25 rEGF*	(5Y)	0.5	a+	p+	a+
26 p gastrin	(1F, 2W)	0.2	p+	a−	p+
27 mGM-CSF***	(6F, 4Y, 1W)	0.5	p+	p+	p+
28 α-lactalbumin	(4F, 4Y, 4W)	0.6	p+	p−	p+
29 b ribonuclease	(3F, 6Y)	0.4	p+	p+	a+
30 p transferrin***	(15F, 12Y, 5W)	0.8	p+	p−	p+
31 b serum albumin	(26F, 19Y, 2W)	0.6	p+	p+	p+

* Rat EGF isolation and sequence determination reported in ref. 24.
** Isolation and protein sequence of GM-CSF reported in ref. 26–28.
*** Isolation and partial sequence determination of p transferrin reported in ref. 25.

biguity arises from the overlap between the second-order minimum for tyrosine at 278 ± 2 nm and one of the local minima of tryptophan at the same wavelength. At tyrosine-to-tryptophan ratios greater than 3:1, the greater contribution from the tyrosine second-order minimum at 280 ± 2 nm adds to the tryptophan local minimum at 278 ± 2 nm to produce a summated minimum at 278 ± 2 nm that is of

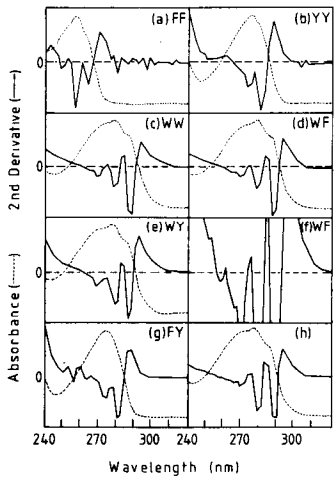


Fig. 1. Zero- and second-order derivative spectra of model synthetic peptides containing different ratios of aromatic amino acids. Each peptide was chromatographed on a Hypersil C_8 microbore column using a linear 30-min gradient from 0 to 100% B where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile–water (60:40). The flow-rate was 100 μl/min and the column temperature was maintained at 45°C. Spectra were acquired during elution of each peptide from the column. Second-derivative plots were obtained at the end of each run. Both the UV-absorbance and the second order derivative have been plotted at 100% full scale, except in the case of panel (f) in which sensitivity has been increased twenty-fold over panel (d). The single letter code is used for the amino acids[31]. (a) FF, (b) YY, (c) WW, (d) WF, (e) WY, (f) WF, (g) FY, (h) YVMGHFRWDRFG.

greater magnitude than the tryptophan minimum at 290 ± 2 nm (see below for examples). Under alkaline conditions, *e.g.* pH 12.5, tyrosine ionises to the phenolate ion which has a second-order derivative plot distinct from that of tryptophan, particularly at 260 nm[15]. However, most reversed-phase supports currently in use are silica-based, having an upper pH limit of 7.5–8.0, which excludes their use at elevated pH. Furthermore such strongly alkaline conditions are unsuitable for peptide and protein purification because of the possibility of chemical modification and polypeptide chain cleavage[29].

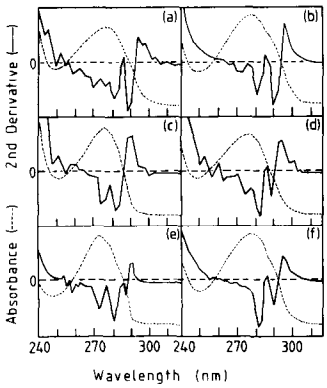
In peptides containing only tyrosine and phenylalanine aromatic residues, such as Phe–Tyr, the spectral contributions of each aromatic amino acid to the derivative spectrum of the peptide can be readily discriminated (Fig. 1g), by the characteristic minimum for phenylalanine at 257 ± 2 nm and for tyrosine at 278 ± 2 nm. Fig. 1h shows the derivative spectra for a polypeptide containing all three aromatic residues (two phenylalanines, one tyrosine and one tryptophan). As in all other peptides examined, the identification of tryptophan by the minimum at 290 ± 2 nm is unambiguous in Fig. 1h. Similarly, the identification of phenylalanine by its minimum at 257 ± 2 nm is unambigous when the attentuation of the derivative plot is decreased twenty-fold. However, the identification of tyrosine in the presence of tryptophan is ambiguous because of the spectral interference from the tryptophan minima. These findings, along with those for other polypeptides, are summarised in Table I.

Spectral analysis of proteins

Spectral analysis of aromatic residues by derivative spectroscopy was extended

to larger more complex polypeptide hormones and proteins using similar chromatographic conditions to those used in obtaining the data in Fig. 1. Examples of derivative spectra of proteins of defined composition are shown in Fig. 2. The identification of tryptophan residues was unambiguous, demonstrated by the derivative spectra of α-lactalbumin (Fig. 2a), gastrin (Fig. 2b), GM-CSF (Fig. 2d), serum albumin (Fig. 2e) and transferrin (Fig. 2f). The unambiguous identification of tyrosine residues depended on the tyrosine-to-tryptophan ratio. For proteins with a tyrosine-to-tryptophan ratio of 1:1, *e.g.* α-lactalbumin (Fig. 2a), the identification of tyrosine was ambiguous due to masking by the tryptophan second-order minima. For those proteins with tyrosine-to-tryptophan ratios higher than approximately 3:1, the deeper minimum at 278 ± 2 nm than that at 290 ± 2 nm identifies the presence of tyrosine residues. Examples of this can be seen for GM-CSF (Fig. 2d) which contains four tyrosines and one tryptophan and serum albumin (Fig. 2e) which contains nineteen tyrosines. In the absence of tryptophan, as in ribonuclease (Fig. 2c), tyrosine can be identified by the minimum at 278 ± 2 nm. Phenylalanine can be identified by its characteristic second-order derivative minimum at 257 ± 2 nm. For example, serum albumin (Fig. 2e) contains 26 phenylalanine residues, clearly identified by derivative spectroscopy in Fig. 2e. In those proteins containing lower molar ratios of phenylalanine, the attenuation of the second-derivative plot needs to be decreased in order to discern the phenylalanine minimum, *e.g.* α-lactalbumin shown in Fig. 2a and GM-CSF in Fig. 2d. The spectral identification of aromatic residues in proteins by derivative spectroscopy is summarised in Table I.

Spectral analysis of three proteins, bovine insulin and rat and mouse EGF, which have similar molecular weight but differ in their aromatic composition, form an interesting comparison. Fig. 3a–c shows the zero- and second-order spectra of



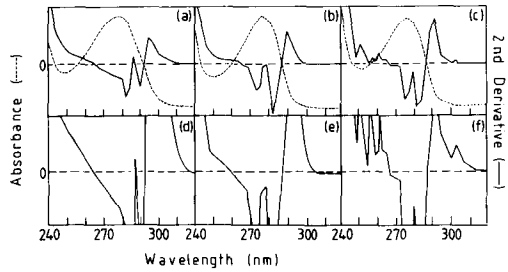


Fig. 2. Zero- and second-order derivative spectra of proteins chromatographed on reversed-phase microbore columns. (a) α-lactalbumin, (b) gastrin, (c) ribonuclease A, (d) GM-CSF, (e) b serum albumin, (f) porcine transferrin. All of the proteins were chromatographed on a Brownlee C_8 short microbore column (30 × 2.1 mm I.D.) using elution conditions identical to those in Fig. 1 except that the column temperature was maintained at 40°C.

Fig. 3. Zero- and second-order derivative spectra of (a) mEGF, (b) rEGF and (c) b insulin, chromatographed on reversed-phase microbore columns. Chromatograpic conditions were identical to those in Fig. 2. (d), (e) and (f), derivative spectra identical to those in (a), (b) and (c) except that the sensitivity was increased twenty-fold.

mEGF, rEGF and b insulin, respectively. The minimum at 290 nm for mEGF (Fig. 3a) indicates the presence of tryptophan. However, the minimum at 280 nm was deeper than that at 290 nm, indicating that the tyrosine molar content was greater than the tryptophan content (see Table I). In comparison, rEGF contains only tyrosine, as indicated by the minimum at 280 nm (Fig. 3b). Zero- and second-order spectra for b insulin clearly indicate tyrosine residues (Fig. 3c) as well as the possibility of phenylalanine. In order to clarify the presence of phenylalanine in b insulin, the derivative spectra for all three proteins were re-plotted at a twenty-fold greater sensitivity. Fig. 3f indeed shows the phenylalanine minimum at 257 nm in the second-order spectrum of b insulin. At this same sensitivity, there were no discernible minima for mEGF (Fig. 3d) or rEGF (Fig. 3e) around 257 nm, confirming the lack of phenylalanine residues. The second-order plots in Fig. 3d and e also demonstrate the lack of interference from the tyrosine and tryptophan spectra in the region of the phenylalanine minimum at 257 nm. In this manner derivative spectroscopy was used to confirm the identification rEGF during its isolation procedure by RP-HPLC[24].

Identification of tryptophan in peptide maps

Spectral analysis of enzymatic or chemical digests of proteins mapped on reversed-phase HPLC allows the unambiguous identification and consequent isolation of tryptophan-containing polypeptides as candidates for amino acid sequence analysis. This is particularly useful when partial sequence data are required for the purpose of constructing high stringency oligonucleotide probes and subsequence cloning of the gene, since the tryptophan DNA codon is unique. This technique is demonstrated in the purification of some of the tryptic peptides of porcine transferrin (Fig. 4). Although many of the eluting peaks in the primary fractionation (Fig. 4) were unlikely to be homogeneous, derivative spectroscopy allowed the identification of tryptophan-containing peaks (marked by arrows) as candidate peptides for further purification and amino acid sequencing. Two peaks, labelled 1 and 2 in Fig. 4, were selected for further purification on the same microbore column but using a different mobile phase composition in order to alter column selectivity[30]. Under the new conditions the change in column selectivity has improved resolution resulting in the further purification of peak 1 (Fig. 4b) and peak 2, which was resolved into two main peak (Fig. 4c). These three peaks were recovered in volumes of about 60 μl and applied directly onto the gas phase sequencer. Amino acid sequence analysis of the three tryptic peptides confirmed the derivative spectral analysis (Fig. 4d–f), which clearly indicated that the three peptides contained tryptophan, no phenylalanine, while the presence of tyrosine would be masked by tryptophan. Using a similar strategy, we were able to identify and ultimately purify for sequencing tryptophan-containing tryptic peptides, as well as other peptides, of the murine transferrin receptor[18] and murine plasma cell antigen PC-1[19]. The resultant partial sequence data were used for the construction of high stringency oligonucleotide probes which enabled cloning of the genes coding for each protein[32,33]. The sensitivity of the method is demonstrated by the final purification and microsequencing of one of the tryptic peptides of mouse transferrin receptor at the 20 picomole level (Fig. 5a). Spectral analysis clearly demonstrated the presence of a tryptophan residue (Fig. 5b), which was confirmed by amino acid sequence analysis[18] (see Table II) and subsequently corroborated by the gene sequence of the cloned transferrin receptor[32]. A recent report has

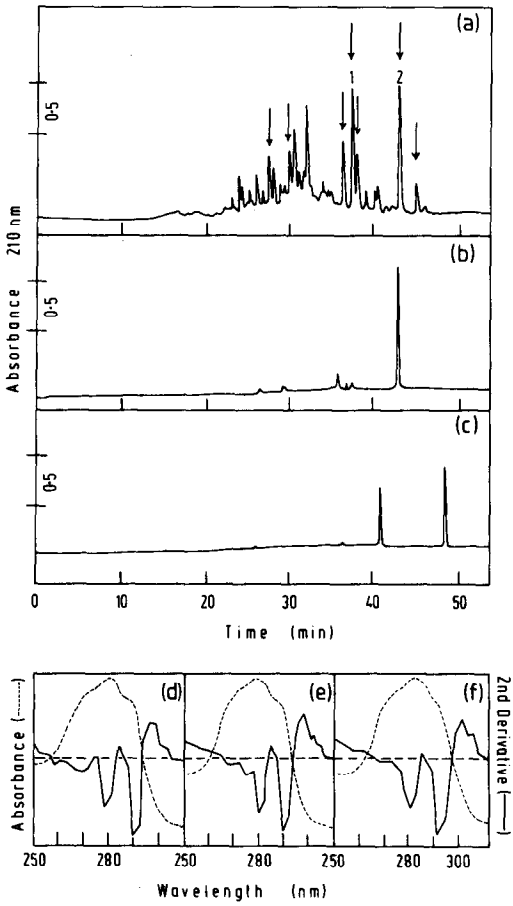


Fig. 4. Purification and spectral analysis of three tryptic peptides of porcine serum transferrin. (a) Primary fractionation of a tryptic digest of reduced and S-carboxymethylated transferrin. Conditions: column, Hypersil C_8; flow-rate, 100 μl/min; linear 60-min gradient from 0 to 100% B where solvent A was water containing 25 m*M* NH_4HCO_3, pH 7.8, and solvent B was acetonitrile–water (50:50) containing 35 m*M* NH_4HCO_3, pH 7.8; column temperature, 45°C; sample load, 25 μg of digest in a volume of 750 μl; detection at 210 nm. UV-absorbing peaks identified by arrows contained tryptophan, as determined by derivative spectroscopy. (b) and (c), Rechromatography of fractions 1 and 2 from (A) above. Conditions: identical to (a) except solvent A was 0.1% TFA in water, pH 2.0, and solvent B was 0.1% TFA in acetonitrile–water (60:40); sample loaded in a volume of 60 μl. Zero- and second-order spectra for the main peak in (b) are shown in (d); sequence analysis of this peak yielded SVDGQEDSIWW-. Zero- and second-order spectra of the peak of 41.5 min in (c) are shown in (e), while those for the peak at 48.5 min in (c) are shown in (f). Amino acid sequence for the peptide in panel (e) was determined as TAGWN-IPMGLLYNK and that for panel (f) was SAGWIXPMGLLYYQLPNK. In all three cases, the minimum at 290 ± 2 nm confirmed the presence of a tryptophan residue.

demonstrated identification of tryptophan residues in tryptic maps separated on standard RP-HPLC columns by using absorbance ratios at discrete wavelengths in the UV-absorbance spectra of eluting peptides[34].

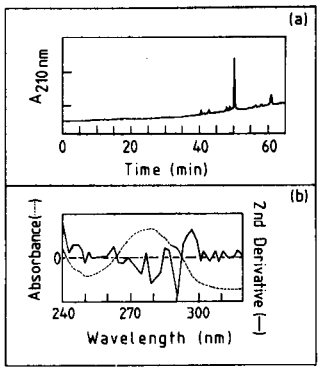


Fig. 5. Final purification and concentration of a tryptophan-containing tryptic peptide from mouse transferrin receptor. (a) One of the tryptophan-containing peaks obtained from the primary fraction of the tryptic digest of mouse transferrin receptor, using chromatographic conditions similar to those in Fig. 3a, was selected for further purification using conditions identical to those in Fig. 4b and c. The main peak was recovered in a volume of 60 μl and loaded directly onto the gas phase sequencer for sequence analysis. (b) Zero- and second-order derivative spectra of the transferrin receptor tryptic peptide. The identification of a tryptophan residue was made on the basis of the second-order minimum at 290 nm, and confirmed by sequence analysis: SIIFASWTAGDFGAVGAT-, as reported[18].

Influence of chromatographic variables on derivative spectroscopy

It is well known that the spectral characteristics of aromatic amino acids are influenced by their local environment[6]. Factors such as pH, organic solvent, denaturants, etc. have been demonstrated to cause shifts in the spectra of aromatic amino acids in peptides and proteins[6]. Indeed, derivative spectroscopy has been used for examining the state of aromatic residues in native and denatured proteins[12,14,16] using non-chromatographic conditions. Peptides and proteins chromatographed on reversed-phase alkylsilicas with gradients in organic solvent concentration are likely to undergo conformational changes[34–36], which could influence the spectral properties of the aromatic residues. Furthermore, spectral shifts may occur depending on the organic solvent concentration at the point of elution of the polypeptide or protein. Any changes in UV-absorbance will be reflected in changes in the intensities and positions of the second-order minima, and potentially could cause the incorrect identification of a particular aromatic residue. We have found this not to be the case because spectral shifts exhibited by fully denatured proteins are of the order of 1–2 nm[14], which is at or below the limit of resolution of the scanning diode array detector used in this study (diode band width of 2 nm). All of the spectra so far examined have demonstrated second-order minima within ±2 nm of the characteristic minima at 257 nm, 280 nm, and 290 nm for phenylalanine, tyrosine and tryptophan, respectively.

Although all of the data reported in this study were acquired on sub-microgram amounts during elution from microbore columns, standard sized columns give similar results when sample amount is appropriately scaled up. Similarly, the method used is compatible with other forms of HPLC separation modes such as ion exchange or size exclusion. The current detection limit for the unambiguous identification of aromatic residues in peptides and proteins is about 10 pmol for a molecular weight of 2000 (approximately 10 ng). Work is proceeding in our laboratory with 1-mm I.D.

microbore columns packed with reversed-phase, ion-exchange, hydrophobic interaction and other types of supports, resulting in a further two- to four-fold improvement in sensitivity because of the reduction in peak volume and consequent increase in sample concentration.

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