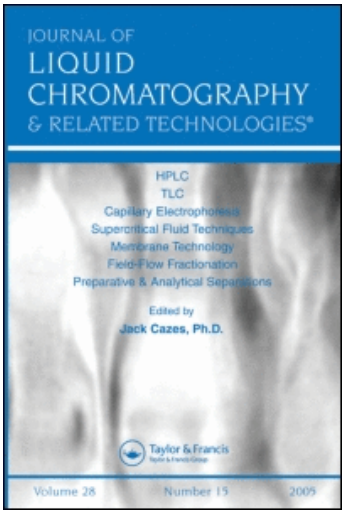

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QUANTITATIVE DETERMINATION OF AROMATIC AMINO ACIDS AT PROTEIN SURFACE BY SIZE EXCLUSION HPLC COUPLED WITH SECOND ORDER DERIVATIVE SPECTROSCOPY

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ABSTRACT

Second order derivative spectroscopy in the ultraviolet (UV) region has the potential to detect subtile differences in the environments of the aromatic residues of proteins. We show here that second order derivative UV spectrum of protein can be a tool to determine quantitatively the aromatic amino acid apparent composition "AAAAC" at protein surface. This non destructive method allows us to obtain conformational information for protein structure-function research and for protein identification in chromatographic process.

INTRODUCTION

Size exclusion high-performance liquid chromatography (SE-HPLC) has been for a long time a widely used technique for both purification and analysis of proteins and peptides. Many applications of SE-HPLC in the research of proteins, such as molecular weight estimation,[1,2] analysis of protein folding[3] and on line conformational monitoring of proteins[4] were investigated. This can sometimes yield many useful informations in protein structure research.

Advances in HPLC photodiode array detection technology have made possible not only to carry out the fly-real time spectral scanning during the chromatographic process, but also to obtain derivative spectra simultaneously.[5-7] Derivative spectroscopy has become a very useful analytical tool to identify and determine aromatic amino acids in proteins and peptides.[8-10]

Derivative spectroscopy offers the advantage of sharper spectral features compared to conventional absorbance (i. e. zero order derivative) spectroscopy. For instance, a peak shoulder present in a zero order derivative spectrum can be transformed into a peak minimum when the second order derivative spectrum is obtained. Furthermore, overlapping band can be transformed into resolved bands.[10] Each aromatic amino acid (Phe, Tyr and Trp) shows a unique near-UV spectrum. Since there is no appreciable peptide bond absorbance above 240 nm,[10] analysis of a second order derivative spectrum from 240 nm to 300 nm shows whether single or multiple aromatic amino acids are present. The determination of three aromatic amino acids in proteins in the presence of denaturant by second derivative spectrometry has been already reported[11-13] and the detection of completely exposed aromatic residues in the denatured proteins was achieved.

In a previous study,[14] we defined the term amplitude as the absorption minima at a specific wavelength for each aromatic amino acid in order to quantify it. Consequently, taking into account that absorption of each aromatic amino acid is additive, one can determine a ratio of one aromatic amino acid to another.[14] Furthermore, the amplitude of minima at certain wavelength is proportional to the amount of each aromatic amino acid. This led us to use free aromatic amino acids as standard to determine the quantity of aromatic amino acids at protein surface by SE-HPLC coupled with on line photodiode array detection. These exposed aromatic residues are often important conformational markers for protein structure function research.[15]

The aim of our paper was to get information about proteins conformations and to apply it for further protein identification during chromatographic process by a non-destructive method.

MATERIALS AND METHODS

All common chemicals and solvents were of analytical grade from commercial sources. Tryptophan, tyrosine, phenylalanine, horse myoglobin (From horse skeletal muscle) and carbonic anhydrase (EC 4.2.1.1. from bovine erythrocytes) were purchased from Sigma Chemicals.

Purification of Yellowfin-Tuna Myoglobin

Myoglobin was isolated from the red skeletal muscle of yellowfin-tuna using a method adapted from Suzuki procedure.[16] The red skeletal muscle of yellowfin-tuna was supplied from Paulet Society (Douarnenez, France). The tissue was homogeneized in distilled water (1:1, w:v) in an ice cold waring blendor. The suspension was then centrifuged (3000 g, 15min., 4°C). The solution was saturated to 60% (w/v) by addition of solid ammonium sulfate.

After centrifugation (3000 g, 15min., 4°C), the precipitate was discarded and the supernatant was saturated to 80% (w/v) with solid amonium sulfate. The new precipitate obtained after centrifugation (3000 g, 15min., 4°C) was dissolved in water and then ultrafiltered and diafiltered (PTGC type membrane, Millipore, 10 KD cut-off) against water.

The crude solution was then loaded on a D.E.A.E Sephacel column (3 cm i.d. x 32 cm.) equilibrated with 50 mM Tris - HCl buffer (pH 8.6). Pure myoglobin was firstly eluted with the same buffer at a flow rate of 60 mL/h. Other impurities were subsequently eluted with the same buffer containing 0.2 M NaCl.[17]

Aromatic Amino Acid Solution Preparation

Tryptophan, tyrosine and phenylalanine were dissolved in 10 mM ammonium acetate buffer pH 6.0 at different concentrations (5.00×10^{-2}, 1.25×10^{-2}, 3.13×10^{-3}, 7.81×10^{-4}, 1.95×10^{-4}, 4.80×10^{-5}, 1.20×10^{-5}, 6.00×10^{-6}, 3.00×10^{-6}M) for calibration curve analyses in SE-HPLC system.

Other solutions of phenylalanine and tyrosine (5.00×10^{-3}M, 5.00×10^{-4} M) and tryptophan (5.00×10^{-4}M, 5.00×10^{-5}M) were prepared to investigate the effect of injected volume on the chromatographic resolution.

Preparation of Protein Solutions

For SE-HPLC analysis, tuna myoglobin, horse mylglobin and carbonic anhydrase were dissolved in 10 mM ammonium acetate buffer pH 6.0 to the respective concentrations (8.28 x 10^{-4}M (14.00g/L), 2.19 x 10^{-3}M (37.00g/L) and 4.66 x 10^{-4}M (13.50g/L)).

HPLC System

The liquid chromatographic system consisted of a Waters 600 automated gradient controller-pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and treat chromatographic data.

Mobile phase for TSK G2000 SWG column (7.6 mm i.d. x 600 mm)

The mobile phase consisted of 10 mM ammonium acetate buffer pH 6.0. The flow rate was 0.75 mL/min. Samples were dissolved in the same buffer, filtered through 0.22 μm filters and injected.

Procedure

In order to investigate response pattern between the second order derivative absorbance of tryptophan, tyrosine and phenylalanine and their amounts in SE-HPLC system, 10 μL and 20 μL of each solution (from 3.00 x 10^{-6} to 5.00 x 10^{-2}M) of aromatic amino acid were loaded on a TSK G2000SWG column.

The other solutions of phenylalanine and tyrosine (5.00 x 10^{-3}M, 5.00 x 10^{-4}M) and tryptophan (5.00 x 10^{-4}M, 5.00 x 10^{-5}M) were loaded on the same column under the same conditions with injected volume from 5 μl to 160 μl to investigate the effect of injected volume on the chromatographic resolution.

5 μL to 40 μL of tuna myoglobin, horse myoglobin and carbonic anhydrase solutions were chromatographed at room temperature on the TSK G2000 SWG column respectively, under conditions described above.

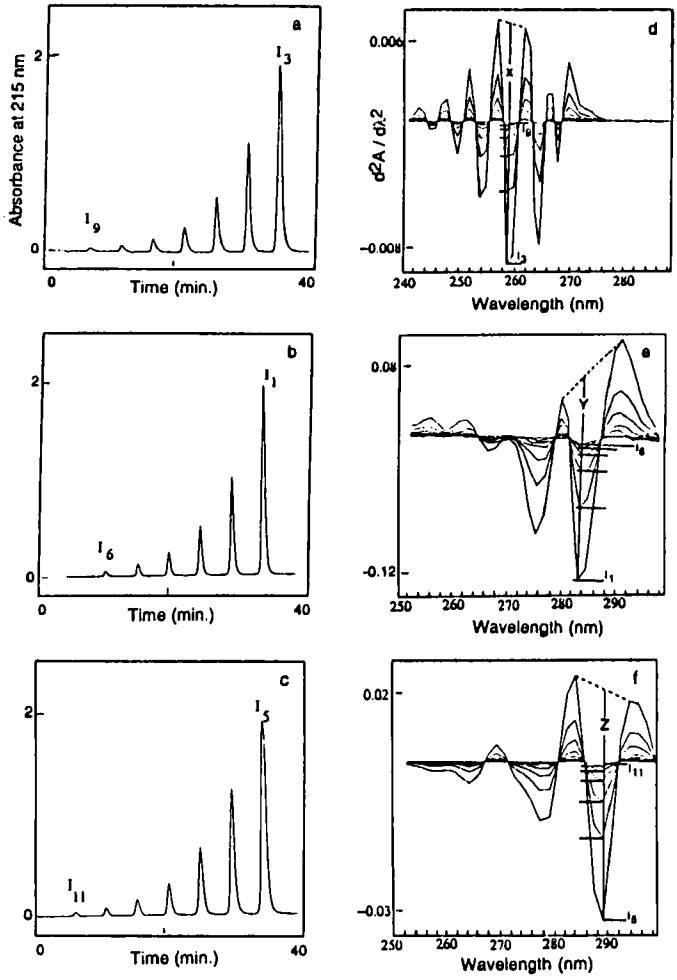

Figure 1. The chromatographic profiles and the second order derivative spectra of phenylalanine (a,d), tyrosine (b,e) and tryptophan (c,f) obtained in SE-HPLC system under the conditions described in experimental section. The amounts and the volume of injection were as follows: I_1 : 1000 nmoles / 20 μL, I_2 : 500 nmoles / 10 μL, I_3 : 250 nmoles / 20 μL, I_4 : 125 nmoles / 10 μL, I_5 : 62.5 nmoles / 20 μL, I_6 : 31.25 nmoles / 10 μL, I_7 : 15.6 nmoles / 20 μL, I_8 : 7.8 nmoles / 10 μL, I_9: 3.9 nmoles / 20 μL, I_{10} : 1.95 nmoles / 10 μL, I_{11} : 0.975 nmoles / 20 μL. X, Y, Z represented the amplitude of Phenylalanine (I_3), Tyrosine (I_1) and tryptophan (I_5).

On-line instantaneous UV absorbance spectral scans were performed between 200 nm and 300 nm with a rate of one spectrum/second for all chromatographic acquisitions. The resolution was 1.2 nm. The results of chromatographic analyses were completed by using Millennium software.

RESULTS AND DISCUSSION

The chromatographic behavior of free aromatic amino acids was investigated firstly. Under our chromatographic conditions, the retention times for the three aromatic amino acids were 31 (Tyr), 32 (Phe) and 35 minutes (Trp) (not shown). Consequently, for each amino acid, series of injections were carried out at the rate of one injection every four minutes. After six or seven injections, the chromatographic and spectral acquisition was started and lasted 40 minutes.

As an example, the peaks corresponding to the increasing amounts injected are displayed in Figure 1 (a,b,c). Their second order derivative spectra between 240 nm and 300 nm are presented on Figure 1 (d,e,f). This indicated that even when the amount of aromatic amino acids changed, the wavelength of inflection point did not vary. This phenomenon was observed and used to determining of aromatic residues in denatured proteins by second order derivative spectrometry.[11]

The same process was repeated to cover ranges between 0.03 and 1000 nmoles (10 or 20 μL injected). As described previously,[14] in second order derivative spectra, X, Y and Z represented the amplitudes of minima at their specific wavelength for Phe (258.5nm), Tyr (283.5nm) and Trp (289.5nm). The amplitudes at their specific wavelength were directly proportional to the aromatic amino acids content. In our chromatographic system (SE-HPLC), free aromatic amino acids were used as standards for calibration curve.

Very good response patterns (Figure 2) are illustrated by the correlation factors r: 0.999 (Phe), 1.000 (Tyr) and 0.996 (Trp). Linear responses were observed from 4 to 1000 nmoles (Phe), 1 to 1000 nmoles (Tyr) and 0.25 to 250 nmoles (Trp) (Figure 2 A, B, C). Identical response patterns for low concentration were displayed in Figure 2 (a, b, c). The slope of the three curves were 6.52×10^{-5} (Phe), 1.60×10^{-4} (Tyr) and 7.26×10^{-4} (Trp) $d^2A/d\lambda^2$ $(nmole)^{-1}$. Both linear responses and curve slopes indicated that the detection sensibility and accuracy for tryptophan is about 4.5 times higher than for Tyr and 10 times higher than for Phe. This is in good agreement with the relative values of the molar extinction coefficient of three aromatic amino acids ($\varepsilon_{Trp} > \varepsilon_{Tyr} > \varepsilon_{Phe}$). These results were very similar to that obtained in simple second order derivative spectrometry.[11]

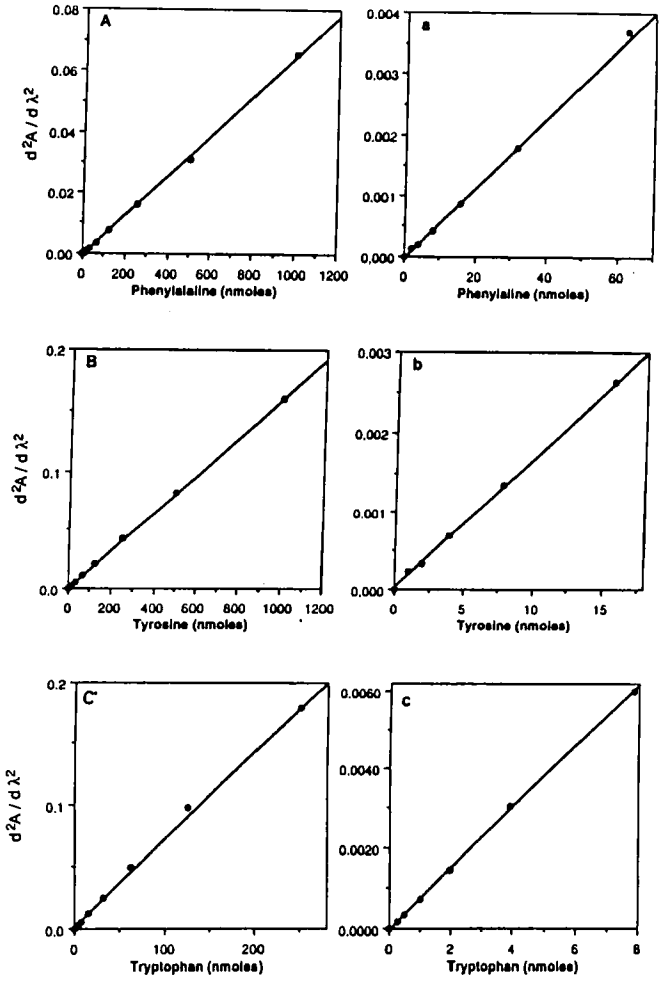

Figure 2. Linear plots of second order derivative absorbances obtained from the analysis on a TSK G2000 SWG column of free aromatic amino acids in SE-HPLC system. The amplitude at 258.5 nm (phe), 283.5 nm (tyr) and 289.5 nm (trp) in great and small quantitiy ranges were displayed for phenylalanine (A, a), tyrosine (B, b) and tryptophan (C, c).

As far as the injection volume is concerned its influence towards chromatographic resolution was demonstrated.[1] In the above results, the volumes of injection varied from 10 μL to 20 μL. Figure 3 (A, B, C) presents

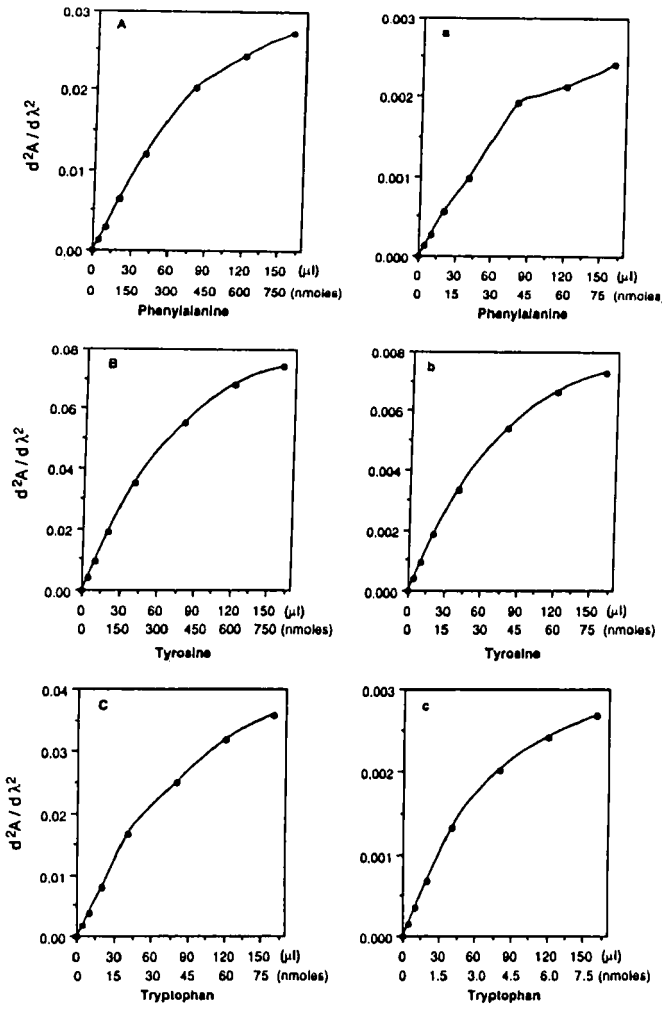

Figure 3. The response pattern between second order derivative absorbances at 258.5 nm (phe), 283.5 nm (tyr) , 289.5 nm (trp) and the injection volume, obtained from the analysis on a TSK G2000 SWG column of free aromatic amino acids in SE-HPLC system. The concentration were 5.00 $X10^{-3}$ M for A and B, 5.00 X 10^{-4} for a, b, C, and 5.00 X 10^{-5} M for c.

the same calibration curve as in Figure 2 except for the injection volumes varying from 5 µL to 160 µL. The quantities of aromatic amino acids in these

volumes were roughly the same as in Figure 2. For injection volume over 60 µL, the linear relation between injected volume of 5.00 X 10^{-3}M (Phe and Tyr) and 5.00 X 10^{-4}M (Trp) solution and amplitudes was no longer verified. This was also observed for ten times lower concentration solutions of aromatic amino acids (Figure 3 a,b,c). As a result to obtain linear response, the calibration must be carried out with small injected volume (below 60 µL).

These optimal conditions for aromatic amino acids were further applied to their determination in native proteins. In this work, acetate ammonium buffer (10 mM, pH 6) was used as mobile phase and gave good resolutions for tuna myoglobin, horse myoglobin and bovine carbonic anhydrase as described below.

The investigation was firstly performed with tuna myoglobin which was extracted from the red skeletal muscle under conditions described in experimental section. Tuna myoglobin which is a heme metalloprotein offered an interesting test case for the second order derivative spectrophotometric analyses, since it showed non-zero absorption in the near UV region.[11] This absorption interferred with the application of the normal absorbance method of Edelhoch[18] for determining tryptophan residues by having broad absorption bands from the visible to UV region.[19] Second order derivative spectrophotometry appeared to resolve these difficulties. Series of injections (5µL to 40µL) of a prepared tuna myoglobin solution (8.28 X 10^{-4}M) were performed on a TSK G2000SWG column. Figure 4 (a) shows one of the chromatographic profiles. Prior to spectral investigations, the purity of tuna myoglobin was checked. A peak integration gave 93% purity for myoglobin. Taking into accounts this results, the real myoglobin concentration was rectified to 7.70 X 10^{-4}M (Table 1).

The known tuna myoglobin molecular sequence comprise one tryptophane, two tyrosine and six phenylalanine residues.[20] Consequently when all aromatic amino acids could be totally detected, the following molar ratios should be verified: Myoglobin/Trp/Tyr/Phe = 1/1/2/6.

Figure 4 (d) shows one second order derivative spectrum of tuna myoglobin. The amplitudes at specific wavelengths allowed us to calculate the amounts of Phe and Trp using free aromatic amino acids calibration curves. The amount of tyrosine was specifically determined by the method previously described.[14] Figure 5 shows the proportionality between myoglobin amount injected and aromatic amino acids quantities detected. The molar ratios for Myoglobin/ Trp/ Tyr/ Phe were determined as: 1.00/1.00/0.57/2.98 [Calculated as follows: $(7.70 \times 10^{-4}/7.70 \times 10^{-4}) / (7.63 \times 10^{-4}/7.70 \times 10^{-4}) / (4.42 \times 10^{-4}$

Table 1

Determination of Aromatic Amino Acids at Protein Surface

		Proteins		
		Tuna Myoglobin	**Horse Myoglobin**	**Carbonic Anhydrase**
Protein concentration (10^{-4}M)		7.7	20.1	3.88
Aromatic amino acids concentration determined by second derivative spectra (10^{-4}M)				
	Phe	23	30.7	17.0
	Tyr	4.42	0.0	0.0
	Trp	7.63	1.17	7.87
Mole of aromatic amino acids for one mole of protein (Theoretical)				
	Phe	6	6	19
	Tyr	2	2	8
	Trp	1	2	7
Mole of aromatic amino acids for one mole of protein (Experimental)				
	Phe	2.98	1.4	4.38
	Tyr	0.57	0	0
	Trp	1	0.53	2.03
Exposure degree of aromatic amino acids at protein surface (%)				
	Phe	49.7	25.5	23.1
	Tyr	29.0	0.00	0.00
	Trp	100	28.2	29.0

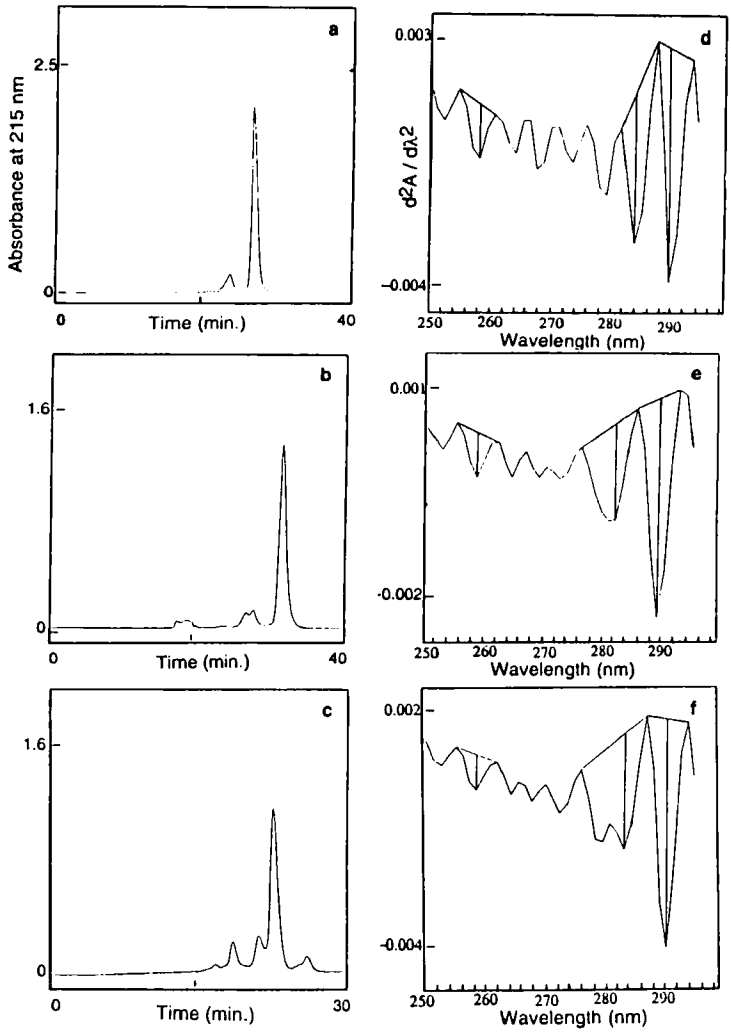

Figure 4. The chromatographic profiles and the second order derivative spectra of 10 μL (prepared concentration: 8.28 x 10^{-4} M) tuna myoglobin (a,d), 5 μL (prepared concentration: 2.19 x 10^{-3} M) horse myoglobin (b,e) and 10 μL (prepared concentration: 4.66 x 10^{-4} M) carbonic anhydrase (c,f) obtained in SE-HPLC system under the conditions described in experimental section.

/ 7.70 x 10^{-4}) / (23.00 x 10^{-4}/7.70 x 10^{-4})]. The comparison of theoretical and experimental molar ratios allowed us to conclude that the aromatic amino acids were not fully accessible to this detection method. Indeed some aromatic amino acids residues were buried inside the protein and not detected, thus the degree of exposure of aromatic amino acids at surface of native proteins could be calculated.[13] Whenever 100% Trp could be detected, the percentage for Tyr and Phe were 28.96% (0.57/2) and 49.67% (2.98/6) respectively (Table 1). Consequently, it could be concluded that the accessibility of aromatic amino acid to the detection method is related to their spacial position in the protein. Thus the more aromatic amino acids are exposed at the protein surface, the more they are detected. On the other hand, the more aromatic amino acids are buried inside the protein, the less they are detected. As far as the tuna myoglobin is concerned, the tryptophan residue is fully exposed at the molecule surface under our non-denaturing conditions. In the same way, globally 28.96% of Tyr and 49.67% of Phe residues are exposed at the surface.

The localisation of aromatic residues within the crystallographic structure of tuna myoglobin[21] confirmed that the aromatic cycle of tryptophan in position 14 was totally situated at the myoglobin molecule surface. The aromatic cycle of tyrosine 21 was partly exposed at the surface whereas Tyr 146 was buried inside myoglobin molecule. As far as phenylalanine is concerned, the classification of the residues with regard to their exposition degree, from totally exposed at the surface to fully buried, was the following: Phe 7 and Phe 151 (100% exposed) > Phe 46 > Phe 43 and Phe 33 > Phe 104 (0% exposed). This information was in very good agreement with the results obtained by our determination (Table 1).

A similar protein, horse myoglobin was then investigated. It contains two tryptophan, two tyrosine and six phenylalanine residues giving a molar ratio for myoglobin/Trp/Tyr/Phe of 1/2/2/6.[22] Figures 4-b and 4-e show the chromatographic profile and second order derivative spectrum. Impurity was calculated by area integration as 8.30% giving a real myoglobin concentration of 7.70 x 10^{-4}M (Table 1). As for tuna myoglobin, the ratios for myoglobin/Trp/Tyr/Phe were measured as 1.00/0.53/0.00/1.40 [Calculated as following: (20.10 x 10^{-4}/20.10 x 10^{-4}) / (11.70 x 10^{-4}/20.10 x 10^{-4}) / (0.00/20.10 x 10^{-4}) / (30.70 x 10^{-4} /20.10 x 10^{-4})]. The aromatic amino acids exposure degrees at the molecule surface were then calculated and displayed in Table 1. It indicated that the two tyrosine residues were completely buried inside the molecule. The two tryptophan residues were globally exposed at 28.51% and the six phenylalanine residues at 25.46%. The crystal structure of horse myoglobin indicated that the localisation of aromatic residue within this molecule[23] had a very good correspondance with our calculated results.

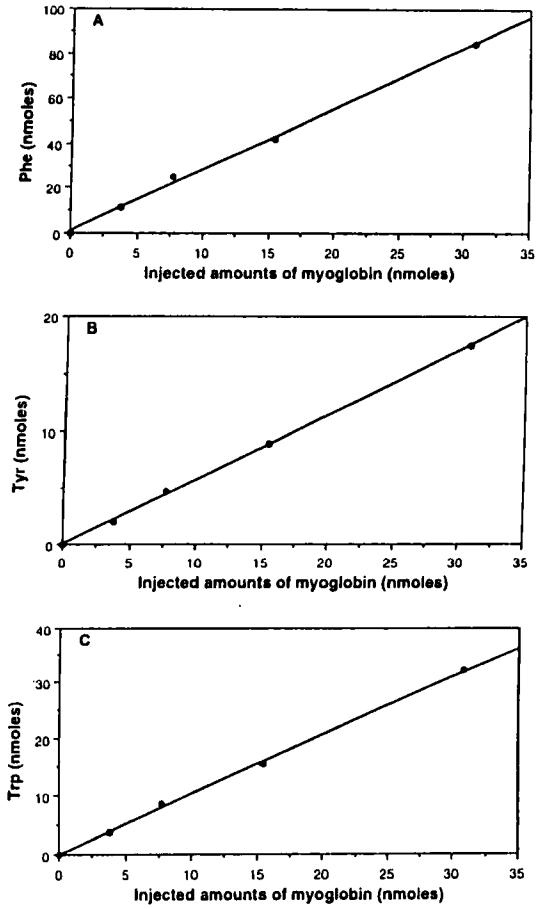

Figure 5. Relation between aromatic amino acid amounts and the different injection quantities of tuna myoglobin in SE-HPLC system under the conditions described in experimental section. The amounts of Phe (A), Tyr (B) and Trp (C) were determined by their second order derivative spectra using free aromatic amino acids calibration curve in Figure 2.

It can be seen that the investigation of horse and tuna myoglobin in SE-HPLC system gave a great difference in exposure degrees of aromatic amino acids at the surface of the molecules. Consequently, these two similar myoglobins could be easily distinguished by the above characteristics.

Carbonic anhydrase (MW: 29000 Dalton) was selected arbitrarily. It contains 7 Trp, 8 Tyr and 19 Phe residues giving a molar ratio for carbonic anhydrase/Trp/Tyr/Phe of 1/7/8/19.[24] Figures 4-c and 4-f show its chromatographic profile and second order derivative spectrum. Impurity was calculated as 16.7% giving a real concentation of 3.88×10^{-4}M. According to their second derivative spectra, the ratio for carbonic anhydrase/Trp/Tyr/Phe were measured as 1.00/2.03/0.00/4.38 [Calculated as following: $(3.88 \times 10^{-4} / 3.88 \times 10^{-4}) / (7.87 \times 10^{-4} / 3.88 \times 10^{-4}) / (0.00 / 3.88 \times 10^{-4}) / (17.00 \times 10^{-4} / 3.88 \times 10^{-4})$]. Exposure degrees of aromatic amino acids at surface of this molecule were calculated as 23.05% (Phe), 0.00% (Tyr) and 28.96% (Trp) (Table 1).

From all above results, the molar ratio between aromatic amino acids at surface of proteins was investigated These calculated ratios are presented in Table 1. These results indicated a conformational state of these proteins in our non denaturing conditions. So it could be postulated that under defined conditions, these ratios were constant and constitute parameter termed as "apparent aromatic amino acid composition" (A.A.A.A.C.) useful for further protein characterization. This parameter is furthermore easily accessible by second order derivative spectroscopy. The fluctuation of this "A.A.A.A.C." for one given protein might indicate a conformational change.[25] It could also be used as a "marker" to identify precisely and follow a protein during a purification process, and assess a protein purity. For example, in spite of very similar amino acid composition and molecular weights, horse and tuna myoglobin are definitely and unambigously distinguishable.

In conclusion, these studies appear to indicate the important role of aromatic amino acid in proteins identification. A method was developed here to determine the A.A.A.A.C. of a protein and characterize it. The conformational change of proteins resulting in aromatic amino acid exposure modification could also be detected by this method. In conclusion, it could be very useful in protein conformation research and for protein structure-function investigation.

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