THE ROLE OF FREE AROMATIC AMINO ACIDS IN THE PROTEIN-LIKE FLUORESCENCE OF WATER NATURAL ORGANIC MATTER

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ABSTRACT

The question about the origin of protein-like fluorescence in the water NOM remains still open because this type of fluorescence may be derived from free amino acids, peptides or proteins contained. The reversed-phase high performance liquid chromatography (RP-HPLC) with multi-wavelength fluorescence detection was used for evaluation the origin of protein-like fluorescence in Suwannee River NOM (SRNOM) and its fractions A, B, C+D, obtained by size exclusion chromatography–polyacrylamide gel electrophoresis setup. The electrophoretic mobilities of fractions varied in the order C+D>B>A and the molecular size (MS) in the opposite order. We demonstrated that the protein-like fluorescence was almost exclusively located in high MS fraction A and medium MS fraction B. Retention times and fluorescence emission spectra of authentic free aromatic amino acids tyrosine and tryptophan were identical with the retention times and emission spectra of some RP-HPLC peaks of fractions A and B. The results obtained for the first time showed that the most part (>50%) of protein-like fluorescence of SRNOM were provided by free aromatic amino acids preserved from further degradation by their incorporation in the SRNOM structure by hydrophobic interactions.

INTRODUCTION

As currently accepted, the fluorescence of water NOM is due to two main groups of fluorophores. One group usually has excitation (Ex) maximum less than 305 nm and emission (Em) maximum less than 380 nm, related to aromatic amino acids, and is often referred to as protein-like fluorophores. The other one with Ex/Em 220–360/380–470 nm is attributed to humic substances (HS)-like fluorophores of water NOM. It is well known, that emission maxima around 280–380 nm correspond to aromatic amino acid residues such as phenylalanine - Phe, tyrosine - Tyr and tryptophan - Trp. Natural protein-like fluorophores in water NOM have been classified by their differences in emission wavelength into tyrosine-like (emission wavelength of around 300 nm) and tryptophan-like (350 nm) types (Coble, 1996; Yamashita and Tanoue, 2003). However, the question about the origin of protein-like fluorescence remains still open because this type of fluorescence may be derived from free amino acids, peptides or proteins.

The objectives of the current work was to use reversed-phase high performance liquid chromatography (RP-HPLC) with multi-wavelength fluorescence detection for comparison of free aromatic amino acids fluorescence with protein-like fluorescence of SRNOM and its fractions obtained by coupling size exclusion chromatography/polyacrylamide gel electrophoresis (SEC-PAGE setup). The multi-step scheme is presented on Figure 1.

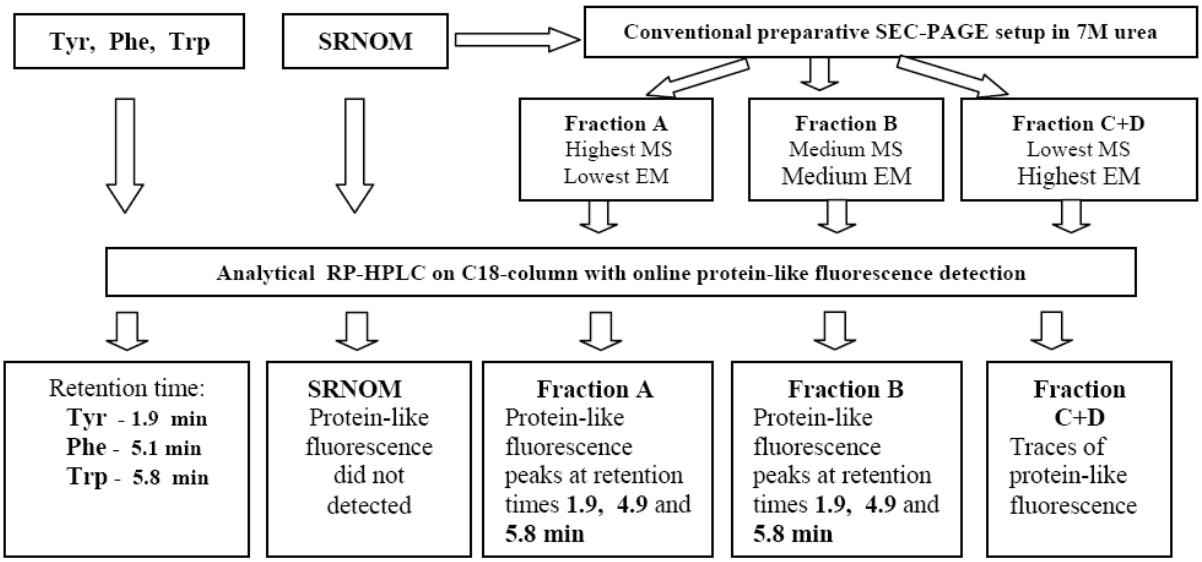

Figure 1. Multi-step scheme of SRNOM preparative fractionation and analysis.

MATERIALS AND METHODS

The SRNOM (ref. number 1R101N), isolated by reverse osmosis from Suwannee River water, Georgia, USA, was fractionated by SEC-PAGE setup (Trubetskoj et al., 1997). The SRNOM fractions named A, B and C+D with different electrophoretic mobilities (EM) and molecular sizes (MS) were obtained (Figure 1). The SRNOM, its fractions and authentic amino acids Tyr, Phe and Trp were analyzed by RP-HPLC on C18-column in 10 mM phosphate buffer (pH 6.5) and stepwise gradient of methanol according to Trubetskaya et al. (2015). A multi-wavelength FLR detector was designed for the excitation wavelength 270 nm and emission range 290-590 nm. To detect peaks with protein-like fluorescence, the RP-HPLC chromato-grams were analyzed at excitation–emission wavelength pairs (Ex/Em) 270/330 and 270/290 nm, because of emission maxima of Phe (278nm), Tyr (305 nm) and Trp (345nm) at pH 6.5. The retention times and fluorescence emission spectra of the RP-HPLC peaks of SRNOM, its fractions and authentic aromatic amino acids were compared.

RESULTS AND DISCUSSION

Setting Ex/Em 270/330 nm and 270/290 nm to detect the fluorescence related to aromatic amino acids, often referred to as protein-like fluorescence, did not reveal essential signals in the bulk SRNOM chromatogram. However, after SEC-PAGE fractionation several additional peaks could be detected on chromatograms of high MS fraction A and medium MS fraction B, where the most abundant were a peak 1 occurring at a retention time of 1.9 min and peaks 2 and 3 at the retention times of 4.9 and 5.8 min, respectively (Figure 1). Low MS fraction C+D contained only traces of these peaks.

To understand the origin of protein-like fluorescence, the RP-HPLC analysis of authentic aromatic amino acids Tyr and Trp was done at Ex/Em 270/330 nm, and Phe – at Ex/Em 270/290 nm, because of amino acid Phe did not emitted at 330 nm. Each amino acid gave one peak, the retention times of Tyr (1.9 min) and Trp (5.8 min) were remarkably similar to those of protein-like peaks 1 and 3, respectively, while the retention time of Phe (5.1 min) was different in comparison with any protein-like peaks.

The fluorescence spectra of peaks 1, 2, and 3 of fraction A and authentic aromatic amino acids were extracted from the data of FLR detector and compared. The fluorescence spectrum of Tyr was very similar to that of peak 1 and fluorescence spectrum of Trp to those of peaks 2 and 3 (Fig. 2). Based on the comparison of retention times and emission spectra it could be suggested that peak 1 represents free aromatic amino acid Tyr (retention time 1.9 min, emission maximum about 300 nm) and peak 3 - free aromatic amino acid Trp (retention time 5.8 min, emission maximum

about 345 nm). The appearance of Trp-like fluorescence in peak 2 (retention time 4.9 min) could be explained by association of Trp with some hydrophilic compounds of SRNOM, which led to a decrease in the retention time from 5.8 to 4.9 min. It was impossible to detect the peak corresponding to free authentic Phe due to its very low absorption at wavelength 270 nm, which has been used in this study as an excitation wavelength, and because quantum yield of Phe (0.04) is one order of magnitude less than that of Tyr (0.21) or Trp (0.17) in neutral pH. The inspection of RP-HPLC chromato-grams of SRNOM and its fractions at Ex/Em 270/290 nm did not revealed any new peaks. The fluorescence emission spectra of peaks 1, 2, and 3 of fraction B demonstrated similar results, but they were not detected in the bulk SRNOM and very poorly presented in fraction C+D. Based on the relative contribution of peaks 1 (corresponded to Tyr) and 3 (corresponded to Trp) in total area of protein-like fluorescence (Figure 2) we could evaluate that fluorescence of free amino acids constitute a major part (more than 50%) of total protein-like fluorescence of SRNOM.

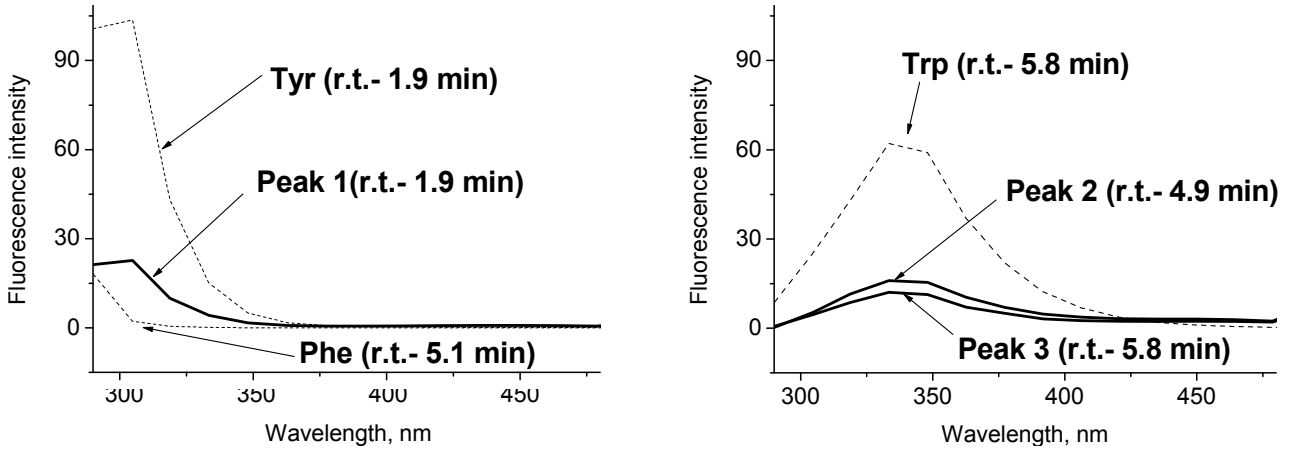

Figure 2: Fluorescence spectra of Trp, Tyr, Phe and RP-HPLC peaks 1, 2 and 3 of high MS fraction A , r.t.-retention time.

Actually, incorporated amino acids species seem to survive the SEC procedure (the free amino acids would elute at the end of the SEC chromatogram and lost during dialysis), but obviously do not survive the RP-chromatography (here they seem to elute at similar retention times as the pure free amino acids).

The presence of incorporated free amino acids in water NOM is extremely important for understanding and verification that water NOM may serve as a reservoir for further using of amino acids in the environment.

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