

Substance P Self-Aggregation Revised: A Chromatographic and Mass Spectrometry Analysis

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Abstract

Size exclusion liquid chromatography and electrospray ionization mass spectrometry have been used for the first time to analyze the possible effect of the environmental conditions on substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂) self-association. The effects of different parameters such as solvent ionic strength, pH and polarity have been evaluated by monitoring the changes observed in aggregation forms registered by size exclusion chromatography. Mass spectrometry has been employed with the aim of identifying the size of the aggregation clusters formed in gaseous state. These two techniques can be considered complementary since they allow describing the same phenomena by different points of view permitting to identify the possible role of environmental parameters in controlling the distribution of different sized aggregates. Thus, new details can be added to the previous results reported by other methods.

The results obtained indicate that substance P was associated under all conditions tested and that very limited variations of the conditions under which substance P was solubilized were accompanied by perceptible changes in the aggregation figures.

Together with existing data, the data obtained have been interpreted in terms of a bi- or multi-stage model according to which an initial stepwise aggregation taking place through successive additions of single molecules is followed by the coalescence of the already-formed aggregates into larger complexes. Such a model appears to be able to explain the large variations in SP aggregation figures, and the high sensitivity of these figures to limited modifications of environmental parameters indicated by the data obtained.

Keywords: Substance P; Aggregation; Environment conditions; Mass spectrometry; Liquid chromatography

Introduction

The first-discovered [1] of the tachykinin family [2,3], substance P (SP), is an undecapeptide characterized by two positively charged and six non-polar amino acid residues. The peptide sequence [4] is such that four of the first six residues (Arg₁, Lys₃, Gln₅ and Gln₆) are polar, and four of the last five (Phe₇, Phe₈, Leu₁₀ and Met₁₁) are non-polar. Since the C-terminal is amidated, the peptide can be rather cleanly divided into a polar, positively charged, N-terminal and a non-polar, and uncharged, C-terminal. Moreover, bulky side chains are present in four of the first six residues (Arg₁, Lys₃ and the two Gln), but in none of the last five.

The possibility of intermolecular (as well as intra-molecular [5]) self-association in polar solvent has been described rather soon [6] after the determination of SP sequence [4]. Sedimentation and quasi-elastic light scattering data measured in water were interpreted by Rueger et al. [7] as indicating self-association with formation of aggregates of two quasi-overlapping size distributions (60-100 and 200-800 nm). The same authors described their circular dichroism (CD) data as strongly influenced by self-association; in addition, CD data recorded with partial C-terminal fragments of SP indicated six residues (Gln₆ - Met₁₁) as the minimum for association, whereas the maximum was found in correspondence with the octapeptide Pro₄ - Met₁₁ (that is, SP devoid of its two positively charged residues). On the basis of infrared spectroscopy (IR) data, self-association was also described by Choo et al. [8], who interpreted their data—recorded a concentration of 10 mg/ml—as indicating that SP aggregates with its terminals aligned together (that is, N with N and C with C). According to Poujade et al. [9], nuclear magnetic resonance (NMR) data indicated that at low ionic strengths substance P aggregated at both basic and acidic—but not neutral—pHs,

whereas at pH 7.4 it only aggregated in the presence of NaCl (on the same topic, see also Chassaing et al. [10]). On the other hand, IR data recorded at neutral pH and in the presence of 150 mM NaCl indicated that substance P was predominantly unstructured, while aggregation was suggested by the spectra recorded in the absence of NaCl at pH 10.0 and 13.0 [8]. Williams and Weaver [11] interpreted Raman spectra recorded in water at high peptide concentration in terms of a family of structures which were reported to be different from those observed in saline solutions. It seems, finally, worth mentioning that, at a concentration of 5 mg/ml, either in water as in both acid and alkaline saline solutions—but not in 50% CH₃CN—SP was described to aggregate into fibrils large enough to be detected by electron microscopy [12].

Evidences of aggregation have also been reported in environments of reduced polarity. Rueger et al. [7] interpreted their NMR data as indicating aggregation in both dimethyl sulphoxide (DMSO) and dimethylformamide, while the possible existence of association equilibrium in DMSO was reported by Chassaing et al. [10]. Toniolo et al. [13], using NMR and IR techniques, described the aggregation of

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SP fragments in a solvent of such low polarity as methylene chloride; the same authors also reported that these aggregates were disrupted by increasing solvent polarity. On the contrary, NMR data obtained by Poujade et al. [9] were interpreted as indicating that SP polymers formed in aqueous environment dissociated by the addition of organic solvents.

As shown by the above-reported data, SP self-aggregation has been extensively studied by spectroscopic techniques, a particularly large array of which has been exploited for this purpose.

On the other hand, liquid chromatography, especially steric exclusion chromatography, appears to be an obvious – although unemployed in the case of SP – technique for analyzing the size distribution of aggregates. Electrospray ionization mass spectrometry (ESI-MS) is a much less used technique for such a kind of analyses: however, it has recently been used to characterize small protein aggregates, and it has been proposed for their semi-quantitative evaluation [14]. The mass spectrometric fragmentation pattern of protonated substance P ions was described to primarily depend on charge status and fragmentation conditions [15], while the aggregation of lysine-containing model peptides was analyzed by ESI-MS by Banerjee and Mazumdar [16] in solvents of different polarity. The data that follow have been obtained by a compound use of these techniques, with the aim of analyzing the size distribution of SP aggregates, and the possible role of environmental parameters in controlling this distribution.

Material and Methods

Sample preparation

Solution buffers and glassware were pre-heated at 37°C. Peptides were dissolved in the appropriate buffer (“solution buffer”) to a final concentration of 1 mM, as determined by ultraviolet absorbance. When indicated, 1×10^5 DPMs of tritiated SP were added to the sample. Before analysis, peptide solutions were maintained at 37°C for the periods of time reported in the text.

Chromatographic procedures

SP purity was verified by reverse phase chromatography on a 4.6×250 mm 5 μ m Nucleodur 100-5 C18 (Macherey-Nagel GMBH, Düren, EC) eluted at 50°C under isocratic conditions at $6.0 \text{ ml} \times \text{cm}^{-2} \times \text{min}^{-1}$ with 25% of CH_3CN in 0.05% trifluoroacetic acid.

Peptide solutions prepared as above (100 μ l) were analyzed by one of the analytical columns described below. Columns were thermostated at 37°C, and the eluent was monitored at 254 nm. When necessary, fractions were collected and either counted for tritium (see below), or pooled, brought to dryness in rotating evaporator and analyzed by mass spectrometry as described under that section.

Diol: A 4.6×250 mm 5 μ m diol (Macherey-Nagel) was equilibrated in 10 mM H_3PO_4 , 100 mM NaCl (resulting pH 2.27) and eluted under isocratic conditions at $6.0 \text{ ml} \times \text{cm}^{-2} \times \text{min}^{-1}$ with the same mobile phase.

Fractogel 40: A 6.0×180 mm Fractogel TSK HW 40 (particle size 25 - 40 μ m, E. Merck, Darmstadt, EU) was equilibrated in 100 mM NaCl buffered at pH ranging from 2.27 to 12.0 with: 10 mM H_3PO_4 (pH 2.27); 10 mM Na phosphate (pH 5 and 7); 10 mM Na borate (pH 8 to 12). The column was eluted at a flow rate of $0.17 \text{ ml} \times \text{cm}^{-2} \times \text{min}^{-1}$ with the same mobile phase.

TSK G2000SW: A 7.8×500 mm 10 μ m TSK G2000SW with a 7.8

$\times 50$ mm pre-column (obtained through Supelco, Sigma-Aldrich, St. Louis, MO, USA) was equilibrated in 0 to 80% (v/v) CH_3CN in 100 mM NaCl, 10 mM Na phosphate pH 7.0. The column was eluted with the same mobile phase at $2.1 \text{ ml} \times \text{cm}^{-2} \times \text{min}^{-1}$.

Peptide determination

The possible correspondence between UV absorbance and SP was verified by the addition of tritiated SP to some of the samples. In this case, fractions were collected; aliquots were transferred to scintillation vials, added of scintillation cocktail (Ultima Gold XR, Perkin Elmer Inc, Waltham, MA, USA) and counted for tritium in a LKB 1219 scintillation counter. In other cases, the presence of SP was verified by mass spectrometry as described below.

Mass spectrometry

Substance P standards solutions of 1 mg/ml in water adjusted at different pHs (2.3, 7.4, 9.0, 12) by addition of 1 M formic acid or 1 M ammonium hydroxide, and in methanol 100%, were analyzed by direct infusion in a mass spectrometer LTQ-XL (Thermo Electron Corporation, CA USA) ion trap with electrospray interface (ESI). Experiments were conducted to adjust the main parameters for optimizing detection of substance P aggregation figures. The following conditions were chosen: nitrogen employed as nebulizer gas: 10 arbitrary units; auxiliary gas: 5; sweep gas: 5; capillary temperature: 200°C; positive ionization mode was achieved by the application of 3.5 kV on the inlet of the mass spectrometer; capillary voltage: 15 V, tube lens: 100 V. The signal at $m/z = 674.5$, corresponding to the double charged entire peptide, was the most abundant one, and was selected for quantitative purpose. Experiments aimed at the recognition of substance P after chromatographic separation were conducted following the procedure optimized in a previous paper [17], and acquiring the signal recorded at 674.5.

Data analysis

Peak width correction: Peak width was corrected for elution volume by calculating the theoretical width of each peak on the basis of the ratio between its elution volume and the elution volume of the first-eluted peak. The “corrected peak width” was then obtained by subtracting the theoretical width from the measured peak width.

Curves were interpolated using the exponential equations $y = a_0 * e^{(a_1 * x)} + a_2 * e^{(a_3 * x)}$ (decay) and $y = x / (x * a_0 + a_1)$ (growth), where e stands for the base of natural logarithms. Equation coefficients were determined by a non-linear iterative procedure (non-weighted Marquardt-Levenberg). The coefficients of the first degree polynomials were calculated analytically using the R package.

Material

Substance P was obtained from Bachem AG (Bubendorf, CH). Tritiated SP was prepared by isotope exchange by rc Tritec AG (Teufen, CH) using as substrate the above Bachem peptide. Solvents for chromatography were chromatographic grade from Romil (Cambridge, UK); all other reagents were of analytical grade and used without further purification.

Results and Discussion

Chromatographic analyses

Substance P was resuspended in solution buffer as described under “Sample preparation”. Aliquots of the resulting solution were analyzed

as described under "Chromatographic procedures".

Ionic strength: To evaluate the possible role of ionic strength in the formation of complexes, the NaCl concentration of solution buffer was varied from 0 to 500 mM, and these samples were analyzed by the diol column described under "Chromatographic procedures". The results obtained (main panel of figure 1a) indicate the presence of diverse components, the relative amount, shape, and chromatographic parameters of which varied while varying NaCl concentration (Table 1). The possible correspondence between ultraviolet absorbance and the presence of SP was verified as described under "Peptide determination": the matching of the data thus obtained with the ultraviolet profiles allows attributing to SP all components shown. Referring to the results obtained at 100 mM NaCl, a major - rather dishomogeneous peak eluted at $K' = 1.61$ and accounting for 92% of the total absorbance - was accompanied by others, the main one eluted at $K' = 3.14$. Shape and chromatographic characteristics of the separated peaks were also modified by rather limited changes of the solution buffer ionic strength: in figure 1b the peaks corresponding to that centered at $K' = 1.61$ at 100 mM NaCl are shown for solution buffer NaCl concentrations of 110, 120, 130 and 140 mM. Since, inhomogeneity is suggested by both shape of the separated peaks and by the irregularities in their chromatographic parameters (Table 1), these peaks were deconvoluted as described under "Data analysis". The insert of figure 1a shows the results of this analysis for the peak eluted at $K' = 1.61$ in 100 mM NaCl solution buffer, which was resolved into five components, while the peak at $K' = 3.14$ (not shown) was resolved into three components.

Since, in the presence of the very polar mobile phase used the diol column should, in principle, operate in steric exclusion mode, the above components could be supposed to represent aggregates of different mass. However, the presence of components eluted at very high K' s

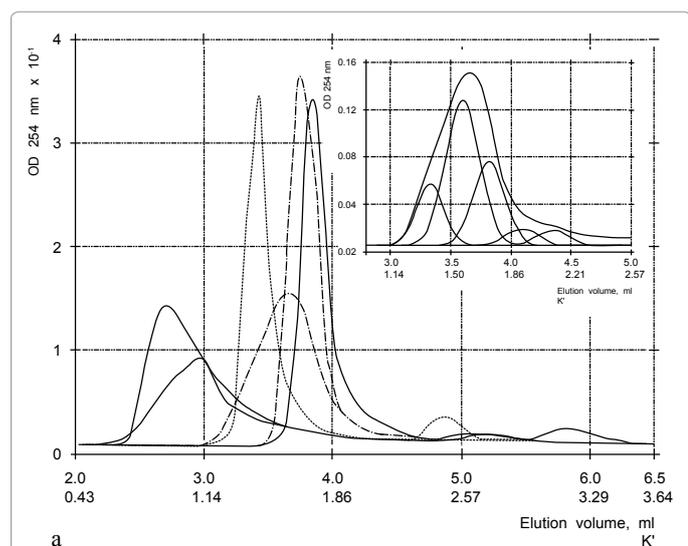


Figure 1a: Main panel: absorbance at 254 nm of the diol column described under "Chromatographic procedures". SP was solubilized in 10 mM H_3PO_4 pH 2.27 plus a NaCl mM concentration of: 0 (thin solid line), 50 (thin dotted line), 100 (thin dash-dotted line), 150 (thick solid line), 200 (thick dotted line) and 500 (thick dash-dotted line). Insert: deconvolution of the peak centered at $K' = 1.61$ in the main panel (solution buffer 100 mM NaCl). Thick solid line represents the original chromatogram, thin solid lines represent the calculated Gaussians, dotted line represents the sum of the Gaussians. Deconvolutions as described under "Data analysis".

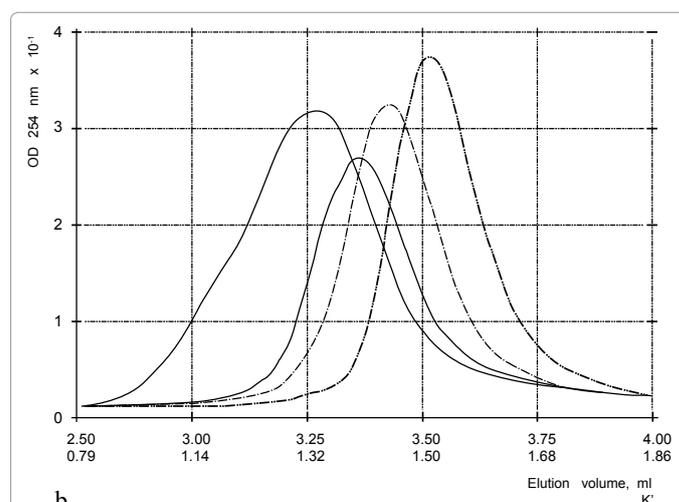


Figure 1b: Detail from 2.5 to 4.0 ml of the column in Figure 1a; solution buffers of NaCl mM concentration of 110 (solid line), 120 (dotted line), 130 (dash-dotted line) and 140 (dash-two dots line) mM. Chromatographic conditions as described under "Methods".

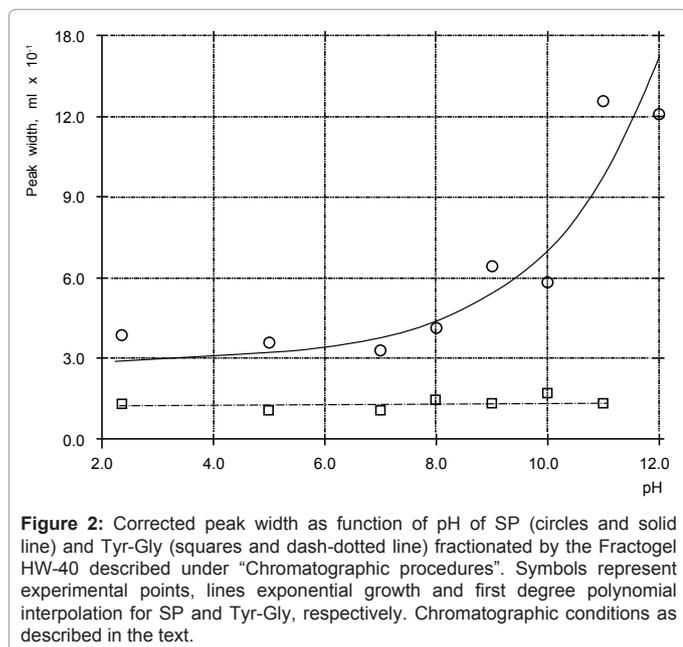
[NaCl]	0.00	0.05	0.10	0.11	0.12	0.13	0.14	0.15	0.20	0.50
K'	0.92	1.11	1.61	1.23	1.31	1.37	1.45	1.75	1.45	1.67
W h/2	0.55	0.53	0.56	0.40	0.27	0.27	0.26	0.22	0.20	0.27
Asmm	3.75	1.57	1.40	1.09	1.54	1.39	1.73	1.29	1.50	1.59

Table 1: Retention factor (K'), width at half height in ml (W h/2) and asymmetry (Asmm) of the main peak in Figure 1a as function of the NaCl molar concentration. Separation conditions as reported under "Chromatographic procedures".

(up to 5.43, not shown) is not compatible with a steric exclusion-only separation, suggesting the co-existence of multiple interactions between SP and stationary phase. As a consequence, while these results can only be interpreted but in terms of multiple aggregates, they make it impossible to identify monomeric SP - if present.

pH: SP was solubilized at pHs from 2.3 to 12.0, as described under "Sample preparation", and these samples were analyzed by the Fractogel 40 described under "Chromatographic procedures". At all pH values, SP was eluted as a single peak (not shown) characterized by retention coefficients between 2.83 at pH 2.3 and 8.35 at pH 12.0. Also in this case, measured K' s are incompatible with a pure steric exclusion separation, suggesting positive interactions between SP and the stationary phase. A plot of corrected peak width, calculated as described under "Data analysis", against pH (Figure 2) was characterized by a large variation interval (337%), and by an exponential dependency. On the contrary, this parameter can be considered constant (Figure 2) for Tyr-Gly. Although the only approximate correspondence between the pK values measured in the free amino acids and their values in structured peptides [18] do not permit a positive attribution of this effect to specific charged groups, the increase of peak width under conditions where SP positive charges are partially neutralized may be interpreted as indicating either a negative effect of charge repulsion on the size dispersion of the complexes, a positive effect of non-polar interactions with the vinyl-based stationary phase (which may increase because of the hydrophilicity decrease), or both [19].

Environment polarity: To analyze this parameter, solution buffer CH_3CN concentration was varied between 0 and 80% v/v (SP solubility limit under the experimental conditions), and these samples were analyzed as described under "Chromatographic procedures".



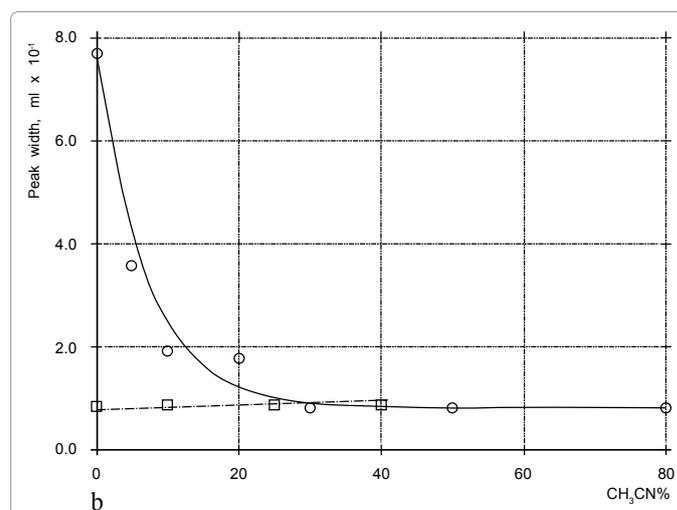
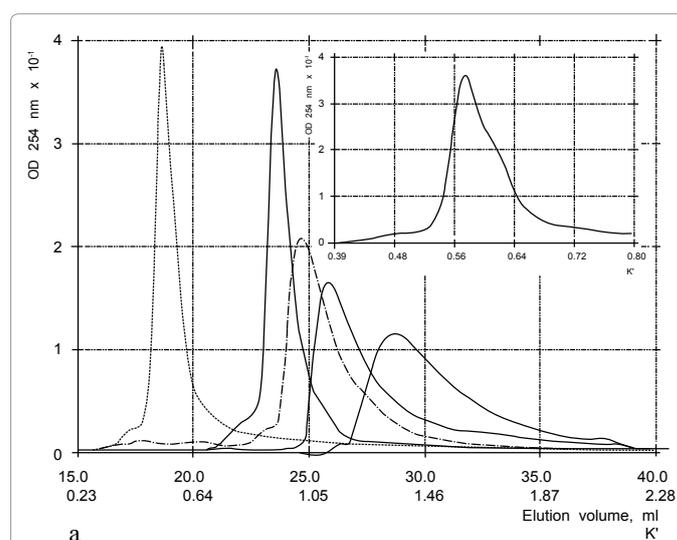
At all CH₃CN concentrations, SP was eluted as a single peak (Figure 3a), showing evidences of in-homogeneity which varied with the CH₃CN concentration. A plot of corrected peak width against CH₃CN concentration (Figure 3b) indicates that this parameter decreased exponentially while increasing the CH₃CN concentration. In the case of Tyr-Gly, peak width was instead constant (Figure 3b) in the same CH₃CN concentration range.

Again, these data may be interpreted as indicating that decreased solution buffer polarity was associated with decreased dispersion of the SP aggregates population, suggesting a positive role of non-polar interactions in complex formation. Alternatively, these data may indicate decreased interactions between SP and stationary phase. Finally, both phenomena may coexist. However, the structure of the stationary phase (silica-based, hydroxyl-derivatized) - not supporting the hypothesis of an effect of solvent polarity on the stationary phase - supports the alternative hypothesis of a negative effect of decreased solution buffer polarity on the dispersion of SP aggregates.

Mass spectrometry analyses

Mass spectrometry was employed to verify the possible presence and size of substance P aggregation figures in gaseous state. ESI allows the detection of solutes with high molecular mass due to online multiple charge ion formation; therefore, it appears to be particularly suitable for the study of peptides aggregates. To evaluate the role of solution buffer on these aggregates, we performed measurements on SP dissolved in aqueous buffers of different pHs (2.3, 7.4, 9.0 and 12.0) and in 100% methanol, as described under "Sample preparation". Solutions were analyzed by direct infusion, acquiring the signals between 600 and 3500 m/z. The mass spectrometer parameters were optimized with the aim to enhance the signals at high m/z, and the signal/noise ratio. In figure 4, the mass spectrum obtained by infusion of the solution of substance P in methanol is shown, and many signals corresponding to aggregation figures are well evident. Two main signals, at 674.5 and at 1349 m/z, representing the double-charged and the mono-charged monomer respectively were observed. In addition, signals at higher m/z value were detected. The most evident of these, the intensity of which was 3% of the intensity of the mono-charged monomer, was recorded

at 2696 m/z, and corresponds to a single-charged dimer. Other signals were recorded at 2022 m/z, corresponding to a double-charged trimer, at 3370, which was attributed to the double-charged pentamer, and at 3146 m/z, corresponding to triple-charged of eptamer. The attribution of the identity and the charge state of these species was performed on the basis of the isotopic distribution. As for signals corresponding to tetramer and hexamer, their respectively double and triple charged species would be equivalent to that of the mono-charged dimer. Thus, these data cannot be interpreted as excluding the presence of these species, since it is possible that some of the detected signals are related to more than one species. On the other hand, isotopic distribution cannot help in discriminating the possible contribution of multiple ions in generating a single signal.



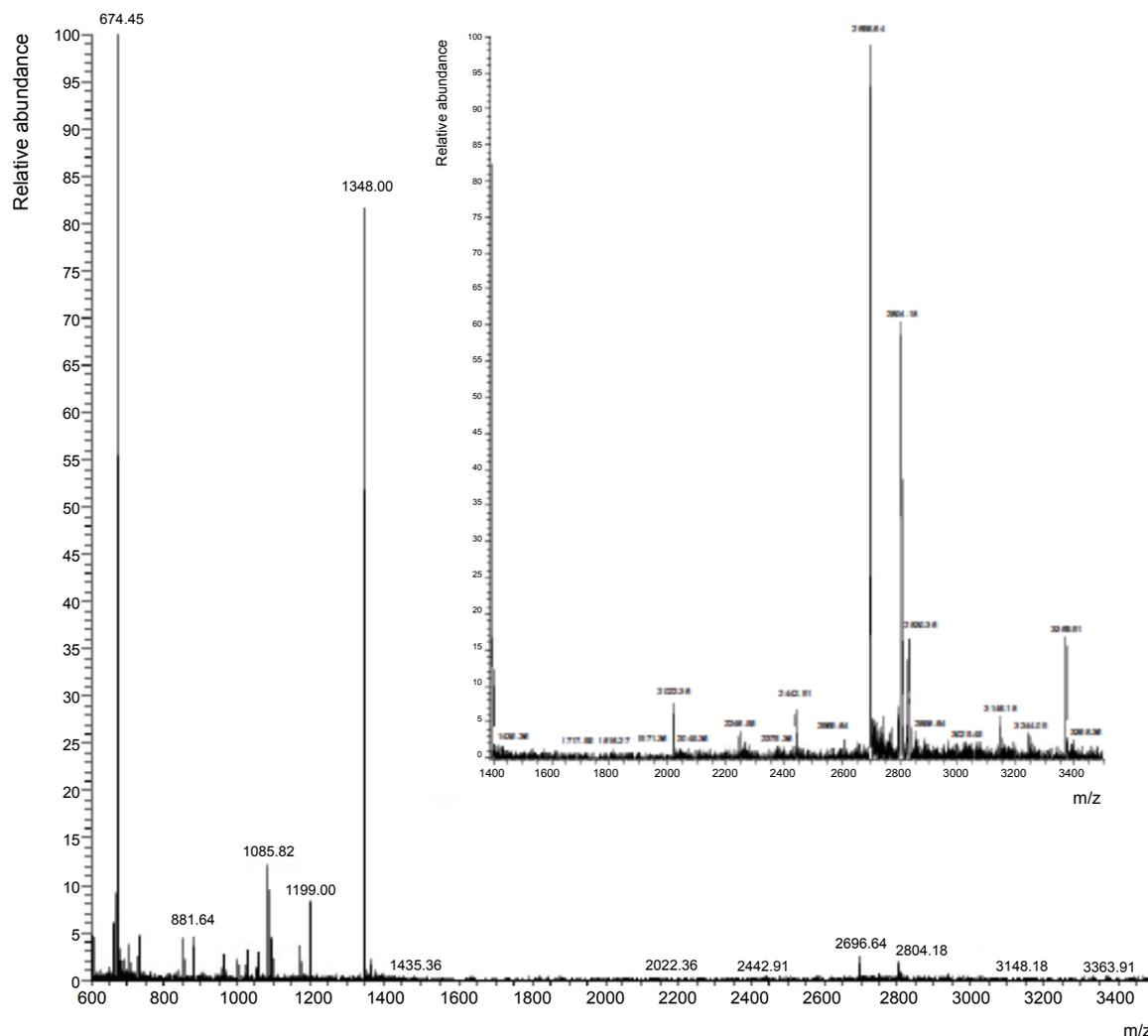


Figure 4: Mass spectrum obtained by direct infusion of a solution of substance P in methanol (1 mg/mL). Main panel: range between 600 and 3500 m/z; insert: range between 1400 and 3500 m/z. Conditions as in the text.

The possible effect of solvent in the formation of aggregates was examined by performing measures in aqueous buffer at different pH from 2.3 to 12. Under all the conditions, the observed pattern was similar to that described for methanol solution: all the above signals were present, but exhibited lower intensity. The mono-charged dimer (2696 m/z) and the double-charged pentamer (3370 m/z) were also in this case the most abundant ones. Among the pH examined, at 2.3 the signal corresponding to the mono-charged dimer was of lower intensity than the signals observed at higher pHs, whereas no significant differences between pH 7.4 and 12 were observed. A semi-quantitative evaluation was performed on the basis of the intensity of the signal of the mono-charged dimer, and results obtained showed a value that in water was found to rise from 1% to 2% for an increase of pH from 2.3 to 12. In methanol, the maximum value corresponding to 3% was recorded. Thus, the decrease in solvent polarity from water to methanol was paralleled by a quantitative enhancement of the signals attributable to SP aggregates. The quantitative dependency of aggregation from pH and solvent polarity suggested by these data could be interpreted as confirming the role of electrostatic interactions in the formation of SP aggregates [16]. The results obtained with methanol indicate the

presence of a higher amount of the mono-charged dimer, and this leads to hypothesize an increase of hydrophobic interactions in this medium with respect to water. Nevertheless, it is necessary to take into account that methanol has a positive effect on ionization [20]; therefore, the concurrence of both effects should be accounted for.

Besides, it is important to underline that all measurements we performed are referred to the behavior of substance P in the gas phase in which the peptide is located after solvent evaporation, and that amino acid and peptide clusters might be formed during the electrospray process [21]. As for the mechanism at the base of the interaction, it has to be taken into account that in gas phase the absence of an aqueous surrounding leads to the disappearance of the hydrophobic effect. Thus, it should be necessary to investigate whether or not the non-covalent complexes that in solution are bound by hydrophobic interactions are preserved in the gas phase [22]. Indeed, studying this phenomenon with model molecules, Barylyuk et al. [22] attributed the existence of complexes in gas phase to the stabilization of Van der Waals forces. In our case, the different intensities recorded for the signal related to the dimer with varying the solvent polarity suggest that some of the interactions existing in the solvent are preserved after evaporation.

Conclusions

The data reported can be interpreted as indicating the presence of un-homogeneous SP aggregates, whose characteristics were found to change upon limited variations of the resuspending buffer. In detail, the ionic strength-associated reduced, and pH-associated increased dispersion of the aggregates reported above can be assumed to indicate a negative role of SP's charges on aggregation. Specifically, the reduction of electrostatic repulsion in the N-terminal region may permit establishing intermolecular interactions which, because of the peptide structure, should involve the non-polar amino acid residues clustered in the C-terminal region. Such an interpretation is also consistent with the higher tendency to aggregate (more exactly, lower peptide concentration necessary for self-aggregation) of the octapeptide Pro₄-Met₁₁ with respect to intact SP described by Rueger et al. [7]. Moreover, as pointed at by Mehliis et al. [6], aggregation may be favored by the decrease of hydrophilicity associated with charge reduction. Data obtained with resuspending buffer of reduced polarity also indicate that decreased dispersion was associated with increased concentration of organic modifier, suggesting a positive role of non-polar interactions on self-aggregation. This interpretation is also consistent with the dis-association of the aggregates formed in aqueous environment following a decrease of environment polarity described by Poujade et al. [9], as well as with the disruption of low environment polarity aggregates achieved by increasing this parameter described by Toniolo et al. [13] for SP fragments.

However, as indicated by our mass spectrometry and chromatographic (not shown) data - and in agreement with the data reported by diverse authors [7,11], although not by Poujade et al. [9] - self-aggregation also occurs at very low ionic strength. Therefore, neutralization of the SP positive charges can be considered as a favoring factor, not a necessary prerequisite for aggregation, thus suggesting that modes of aggregation may be different at low and high ionic strength.

Indicating the presence of aggregates stepping up by single monomers, and in accord with the model proposed by Rueger et al. [7], mass spectrometry data are consistent with the building of aggregates by successive additions. On the other hand, chromatographic data - indicating the existence of discrete aggregates, or groups of aggregates - are not consistent with the hypothesis of a continuum ranging from single monomers to the fibrils visible by electron microscopy [12]. A hypothesis capable of explaining both sets of data consists in the initial formation of small aggregates, followed by their successive coalescence into larger aggregates. In these processes, the formation of hydrogen bonds [6-8,13] should also be taken into account: although the stability of aggregates to heating [7] suggests a comparatively limited role of hydrogen bonds in aqueous environment, their relevance should increase at low polarity. Thus, it can be hypothesized that the interplay of these three factors - reduction of electrostatic repulsion, non-polar interactions and hydrogen bonds - variable upon different environment conditions, may be responsible for the multiplicity of aggregation figures evidenced by the data shown.

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