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Experimental study on vasoactive intestinal peptide (VIP) and its diaminopropane bound (VIP-DAP) analog in solution

Review Article

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Summary. Bioactive peptides represent an exciting area of research in the fields of biochemistry and medicine and in particular the VIP/PACAP network appears to be of interest. Vasoactive intestinal peptide (VIP) is a pleiotropic factor that exerts a physiological regulatory influence and is involved in the pathogenesis of several human disorders. In this paper we have reported structural characterization of VIP by experimental and computational methods as well as a comparative analysis of the peptide with its transglutaminase catalyzed analog VIP-Diaminopropane (VIP-DAP).

Keywords: Vasoactive intestinal peptide (VIP) – Transglutaminase (TGase) – Limited proteolysis – Computational study – VPAC receptors

Abbreviations: cAMP, cyclic AMP; CBP, CREB binding protein; CRE, cAMP responsive element; CREB, CRE-binding protein; CyRE, cytokine response element; GAS, gamma activated sequences; IFN-γ, interferon gamma; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; IRF-1, interferon regulatory factor 1; LPS, lipopolysaccharide; PACAP, Pituary adenylate cyclase-activating polypeptide; PKA, protein kinase A; TNF-α, tumor necrosis factor α; VIP, vasoactive intestinal peptide

Introduction

Bioactive peptides are an exciting area of research in the fields of biochemistry and medicine. Peptides are small molecules of living organisms that influence cellular communication through binding and activation of specific receptors endowed in the plasma membrane. These molecules control a wide spectrum of life functions as neurotransmission, metabolism, electrolytic levels, immunity, inflammation, digestion, respiration, pain sensitivity, reproduction, etc. Endogenous and/or exogenous bioactive peptides released into the organism act as regulators with hormone-like activity. It is intriguing to study the details of the molecular mechanism underlying the role played by a variety of these molecules in the regulation of the inflammatory and immune responses. In fact, it could be useful for clinical applications the knowledge of the biochemical homeostatic response of human organisms to damaging physical, chemical or biological stimuli. Among these peptides, VIP/PACAP network appears to be of interest (Arimura, 1992; Gomariz et al., 2001). VIP was first isolated from the small bowel (Said et al., 1970) and was later found in the central and peripheral nervous systems as a neuropeptide with wide distribution, acting as a neurotransmitter or neuromodulator in almost all organs and tissues. VIP is a pleiotropic factor that exerts a physiological regulatory influence on a wide range of body functions (pancreatic and intestinal secretion, regulation of smooth muscle motility, gastrointestinal blood flow, mucosal immune function, inflammation, etc.) and is involved in the pathogenesis of several human disorders (Fahrenkrug and Said, 2000). Two carboxyamide molecular forms, PACAP 1–27 and PACAP 1–38, were first isolated from sheep hypothalamic extracts (Miyata et al., 1989). The two forms share an identical 1–27 amino acid sequence that exhibits 68% similarity with VIP; PACAP-38

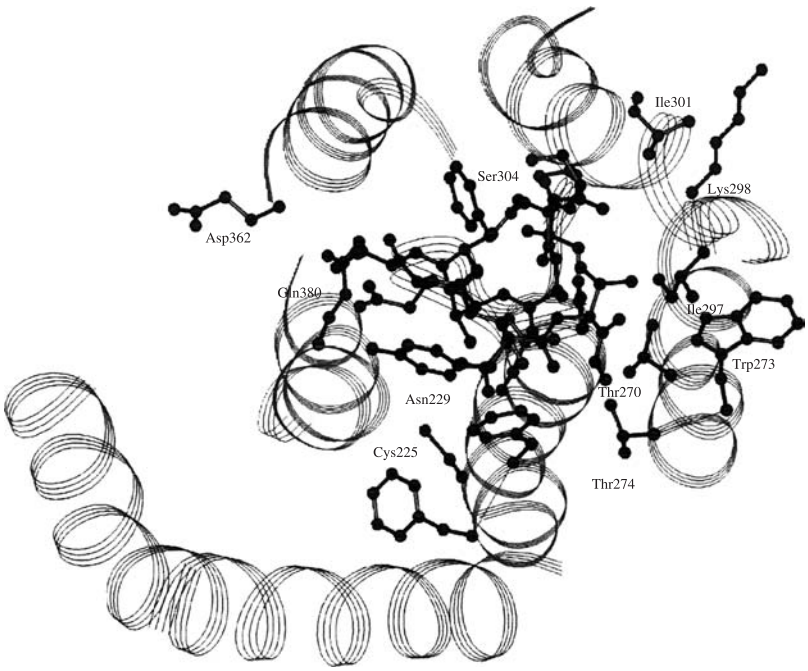

Fig. 1. 3D structure of the refined model of the VIP (1–11)/VPACI receptor complex. Relevant amino acid residues involved in the interaction with the ligand are displayed. This model has been gently granted by Dr. Marta Filizola

represents 80–90% of the total PACAP content in all tissues studied (Christophe, 1993). Both peptides activate pituitary adenylate cyclase and their effect has been identified in nerve fibres, in the gut wall and submucous and myo-enteric ganglia of several species, including humans (Fahrenkrug and Said, 2000). Although PACAP physiological role has not yet been clarified, its equipotent affinity to a VIP receptor indicates an activity profile identical to that of VIP (Christophe, 1993). The respective physiological roles of VIP and PACAP in a given tissue expressing VIP receptors depend on the relative amount of VIP and PACAP secreted in the vicinity of the receptor and on the relative stability of the peptides (Gourlet et al., 1997). It has recently been shown that PACAP is a potent relaxing agent on several tracheal or bronchial preparations, as efficient as VIP (Christophe, 1993; Gourlet et al., 1997). Moreover, in some preparations, PACAP exerted a more sustained effect. It was also suggested that the prolonged action of PACAP-38 might result from its poor degradation by neutral endopeptidase 24.11 (NEP), an endopeptidase present on lung epithelial cells and involved in the inactivation of peptidic hormones (Gourlet et al., 1997). To test this hypothesis, the degradation of VIP, PACAP-27 and PACAP-38 by purified neutral endopeptidase prepared from human kidney, was compared: both VIP and PACAP-27 were cleaved by NEP, but PACAP 38 was not (Gourlet et al., 1997). The VIP and PACAP biological effects are exerted through a family of receptors represented by VIP/PACAPI or VPAC1 (Fig. 1), VIP/PACAP 2 or VPAC 2, and PACAP 1 or PAC 1 (Harmar et al., 1998), which transduce the ligand signal through the activation of different enzymatic effector systems, such as adenylate cyclase, phospholipase C, and iNOS (Louis et al., 1988; Leceta et al., 2000). VPAC 1 and 2 exhibit similar affinities for the two neuropeptides and activate primarily the adenylate cyclase system. VIP receptors are coupled through a Gαs protein to a PKA signalling pathway in human and rodent immuno-competent cells as well as in several cell lines (Calvo et al., 1996). The mitogenic stimulation of cell suspensions from different lymphoid organs was found to elicit the secretion of VIP (Pozo et al., 2000). In endocrine and neuroblastoma cells VIP secretion is regulated by cyclic AMP (cAMP), Ca^{2+}, protein kinase C (PKC) and cytokines; a cAMP response element (CRE) and a cytokine response element (CyRE) have been located in the VIP gene promoter (Waschecck, 1994). These mechanisms might also regulate the expression of this neuropeptide in immune cells, since interleukin 1 (IL-1), IL-6, and tumour necrosis factor α (TNF-α) are potent inducers of its secretion (Martinez et al., 1999). The VIP/PACAP receptors belong to the receptor family known as G-protein coupled receptors (GPCRs), a large group of integral proteins of pivotal importance, directly involved in the transmission of signals inside the cells. They consist of a single polypeptide chain containing seven hydrophobic domains looping back and forth across the phospholipidic layers of plasma membrane (Strader et al., 1994; Watson and Arkinstall, 1994). Analysis of

the hydrophobicity profile of these transmembrane domains computed from their amino acid sequence suggests that they adopt helical secondary structures (Samatey et al., 1995). These helices pack in a bundle, arranged to exhibit a hydrophobic outer surface facing the lipidic membrane and a hydrophilic inner surface that originates a binding pocket in the interior. In these receptors the seven transmembrane helices as well as the extracellular loops are involved in peptide binding. On the other hand, intracellular loops are coupled to a G-protein and are implicated in the transduction of the signal. Some of these receptors, such as the VIP-secretin receptor family, exhibit highly restricted G-protein coupling. The G-protein mediate signal transduction through a well known GTPase cycle (Birnbaumer, 1990). The effector molecules coupled to G-protein are integral membrane proteins (i.e., adenylate cyclase or ion channels) or membrane phospholipids (e.g., phospholipase C). They generate intracellular second messengers, such as inositol triphosphate (IP3) and cyclic AMP that are released into the cytoplasm to activate exocytosis or modulate the activity of many transcription factors. This coupling depends on the specificity of protein–protein interactions among the receptor, G-protein, and effectors. Defining these interactions, it is possible to provide a potential target for the discovery of new therapeutic molecules that specifically interfere with cellular signaling events.

Bioactive conformation of VIP

The study of the structure-function relationships of this peptide is important in order to characterize its interactions with the receptors and to modulate its stability and affinity. Several efforts have been made to identify the preferred conformation of VIP in solution, with different techniques, and to date several controversial reports can be found. A conformational study explored the theoretically preferred conformation of VIP by combining experimental information with unrestrained molecular calculation. The only structural information available on the peptide has been mainly obtained by circular dichroism (CD) and nuclear magnetic resonance (NMR) analysis

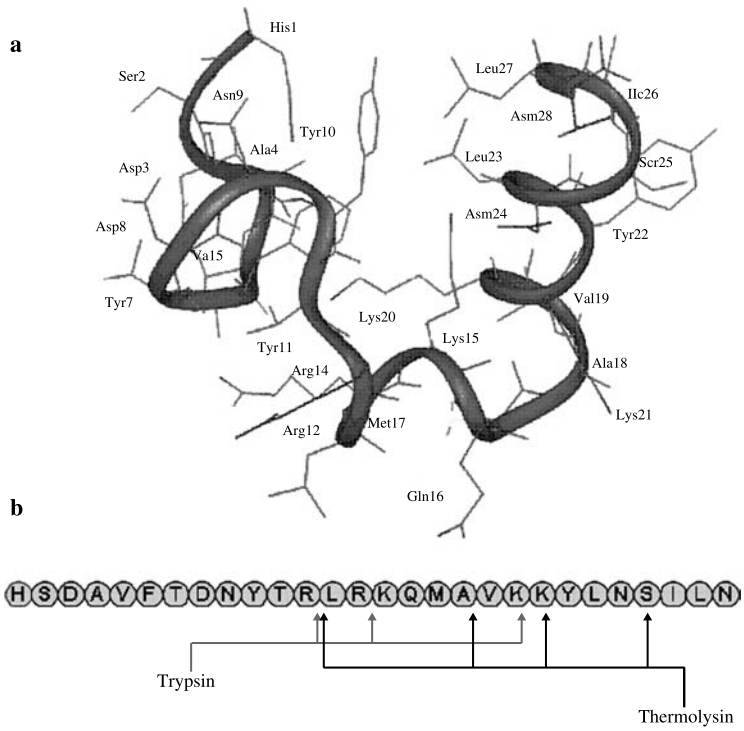

Fig. 2. **a** 3D structure of VIP. Ribbon representation of the energy-minimized starting structure of VIP. Minimization was carried out using the AMBER 4.0 program in two steps. First, the initial structure of VIP was energy minimized in vacuo using a dielectric constant of 4r and imposing restraints on the distances between two pairs of residues, in order to force the coiled coil structure at the C-terminus. The distance between the Cα atom of Lys15 and the Cα of Lys20 was set to 5.0 Å, whereas the distance between the Cα atom of Tyr10 and the Cα of Leu23 was set to 8.0 Å. The structure was then subjected to a conjugate gradient minimization until convergence was achieved, when the rmsd of the gradient was less than 0.001 Å. In a second step the peptide structure was immersed in a box of 2352 TIP3P water molecules and it was completely relaxed. A new conjugate gradient minimization was then carried out using a dielectric constant of 1r until convergence was achieved (rmsd <0.001 Å). **b** Linear aminoacidic sequence of VIP. The arrows indicate the hydrolysis sites after 5 min of incubation with Trypsin and Thermolysin (enzyme:substrate ratio 1:1000)

(Theriault et al., 1991). The main limit of all these studies derives from the fact that they have been performed in the presence of a high concentration of helix-inducing solvents or other non-physiological solvents.

Therefore, more experiments are required to define the conformational features of VIP in solution under physiological conditions. In our recent paper we utilized the limited proteolysis to provide structural information of peptide in solution in a situation close to physiological conditions. The results suggest that the peptide chain is more flexible near positions 12 and 21/22 of the peptide sequence, the N- and C-terminal regions are compact and the presence of secondary structures confer a rigidity thought proteinases' attack. Moreover a hinge region is present in the middle of the peptide sequence. Further ES-MS analysis of VIP have been shown that Met^{17} residue is partly oxidized and clearly accessible to the solvent. These results agree with NMR studies on VIP and VIP analogues that proposed a molecular model in which the N-terminal portion could have bent or β-turn structures stabilized by hydrogen bonds (Filizola et al., 1997; Stiuso et al., 2006).

In Fig. 2 was reported the energy minimized structure of VIP consistent with all available structural information (NMR and computational studies) (Theriault et al., 1991; Goossens et al., 1996) and guided by limited proteolysis results (Stiuso et al., 2006). The model was obtained with AMBER 4.0 program according to following procedure: 1) the N-terminal segment was added to this structure using the geometry of the lowest energy conformation found for VIP (1–11) in our previous study, and was also added not exposing to the solvent residue Tyr^{10} that is never hydrolyzed by limited proteolysis; 2) dihedral angles of a regular right-handed α helix ($\phi = -57°$, $\psi = -47°$) were assigned to residues on segments 12–15 and 19–28, according with NMR results (Watson and Arkinstall, 1994); 3) dihedral angles of the central segment (residues 16–18) were manually set to adopt an initial folded conformation in such a way that side chains of specific residues were exposed or hidden according to the information deduced by the limited proteolysis experiments. Stability of the coiled-coil conformation at the VIP C-terminus was investigated by an extensive dynamics simulation of 500 ps at a constant temperature of 300 K, with the peptide soaked in a bath of TIP3P water molecules. The results show that the coiled-coil conformation is not stable after the first 150 ps and the two helices open providing a structure with two α-helices joined by a flexible region as suggested in previous NMR studies (data not reported). The solvent exposed area of different residues involved and not in the proteolytic experiments was computed along the dynamic process as showed in Figs. 3 and 4. Figure 3 clearly shows that Tyr^{10} and Lys^{20} become more solvent exposed than in the initial model of VIP, while the exposure of Lys^{15} remains the same during the dynamics process. On the other hand, Fig. 4 shows that Arg^{12} and Arg^{14} appear less exposed in respect to their initial positions, while

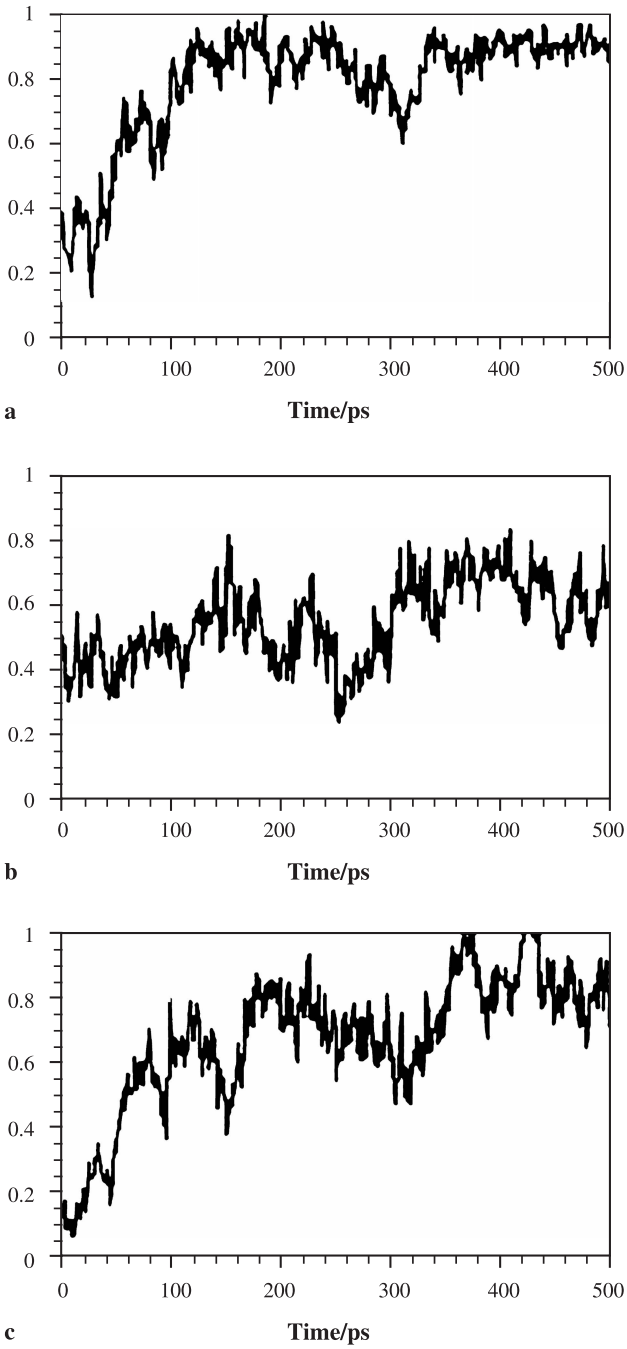

Fig. 3. Percentage of the solvent-accessible surface of different non-exposed residues: **a** Tyr^{10}; **b** Lys^{20}; **c** Lys^{15}

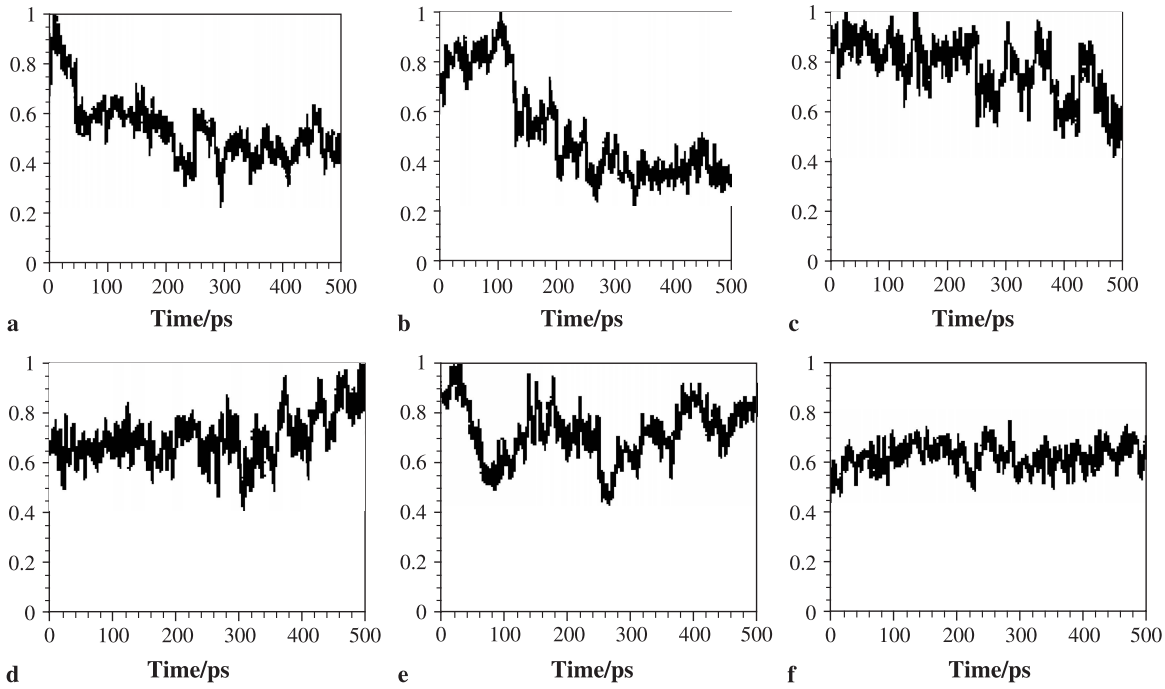

Fig. 4. Same as in Fig. 3 for those residues solvent exposed: **a** Arg^{12}; **b** Arg^{14}; **c** Gln^{16}; **d** Met^{17}; **e** Lys^{21}; **f** Tyr^{22}

Gln^{16}, Met^{17}, Lys^{21} and Tyr^{22} had been directed correctly. Lys^{15} remains hidden to the solvent during all the dynamics process due to a hydrogen bond with the carbonyl oxygen of the Arg^{12} backbone. In contrast, Tyr^{10} and Lys^{20} appear to be totally exposed to the solvent. Interestingly, these two residues exhibit water molecules acting as bridges between each of them and neighbouring residues. Specifically, Tyr^{10} exhibits a water molecule acting as a bridge between this residue side chain and Lys^{15}. Similarly, Lys^{20} is involved in a hydrogen bond with a water molecule that forms a bridge with Gln^{16}. In this way, residues although exposed to the solvent, appear hidden to the more voluminous enzyme avoiding the bond cleavage at this position. These results on VIP propose a molecular model in which the N-terminal portion could present bent or β-turn structures stabilized by hydrogen bonds (Nelson and Kallenbach, 1986). Moreover this peptide in solution and in the absence of structuring solvents is characterized by the presence of some segments with secondary structure, linked together by "hinge" regions that confer flexibility to the peptide. These hinge regions seems to be located in positions 12–14 and 21–22 of the sequence. The sites of proteolysis are in regions that fold into a helical structure only when the peptide is treated with helix-inducing agents. On the other hand, regions resistant to proteolysis are located also in portions of the peptide previously considered as not structured (Lehrman et al., 1990).

Comparative analysis of VIP and its analog

The VIP N-terminal helix is known to be critical for the high affinity binding and coupling to the effector system, while the C-terminal sequence has been shown to be important for VPAC 1 and 2 discrimination (Couvineau et al., 1984; O'Donnell et al., 1991; Ciccarelli et al., 1994; Wulff et al., 1997). Concerning the central region of the VIP polypeptide chain, different amino acid substitutions at this site did not affect the affinity or activity of this bioactive peptide, suggesting that this region is not directly involved in the recognition or activation of receptors. In contrast, Robberecht and his collaborators demonstrated the unexpected importance of Gln16 in the central region of the secretin family peptides in the interaction with the receptor N-terminal domain (Gourlet et al., 1996). On the basis of this finding, we were previously prompted to use the transglutaminase (TGase) enzyme to modify the Gln^{16} of VIP with a variety of amines of different carbon chain length and positive charge in order to investigate the effect of these changes on the interaction of the peptide with VPAC receptor and on its subsequent activation (Lorand and Conrad, 1984; Porta et al., 1988, 1990; Aeschlimann and Paulsson, 1994; Esposito et al., 1995). Only the VIP-1,3-diaminopropane (VIP-DAP) act as agonist with both a higher affinity and a higher maximal effect than VIP and stimulate the VPAC 1 receptor.

This finding supported Robberecht's group data pointing out to the critical role played by the occurrence of a positively charged amino acid (arginine = R) at position 16 of VIP polypeptide chain (Gourlet et al., 1996). The high affinity of VIP-DAP might be related to specific interactions of this agonist with well defined hydrophilic regions of the receptor polypeptide chain. The analysis by limited proteolysis of VIP-DAP demonstrated that the derivatization of Gln^{16} is able to protect Met^{17} from oxidation, as shown in Fig. 5, Arg^{12} from hydrolysis and to improve the resistance to the proteolytic attack (Stiuso et al., 2006). Experiments are in progress to analyze the antioxidant and proliferate role of VIP-DAP in biological systems.

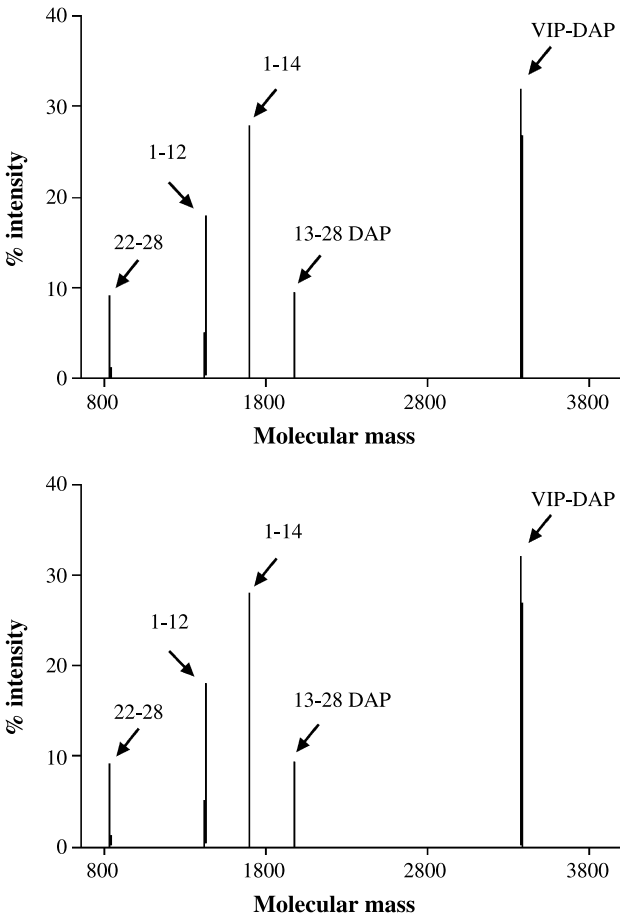

Fig. 5. Deconvoluted ES Mass spectra of VIP and VIP-DAP fragments performed at 20–22 °C utilizing trypsin as protease: substrate ratio 1:1000. Electrospray mass spectrometry (ES-MS) analysis of peptides was performed by a Q-TOF mass spectrometer (Waters/Micromass, UK Ltd., Manchester, UK). The spectra were obtained by scanning from m/z 2000 to 400 at 2 sec/scan. Mass scale calibration was carried out using the multiple charged ions from a separate injection of myoglobin. The amount of the various components was assessed by integration of the intensity of the multiply charged ions of the single molecular species

Concluding remarks

The possible use of bioactive peptides in the treatment of a variety of important chronic diseases such as infections, asthma, reumathoid arthritis, sarcoidosis, connective tissue disorders, etc., has recently stimulated a variety of studies investigating the relationships between structure and molecular mechanism action of these potential drugs. Structural-activity studies, performed on a number of analogs and different VIP fragments, demonstrated that full action of VIP is critically dependent upon integrity of the entire molecule. Novel chemical modifications of exposed solvent residues, similar to those reported in this paper, could be driven to the synthesis of agonists able to discriminate between receptor subclasses for a selective control of biological functions and to guarantee the stability to protease-s'attacks. The future research will be directed by an interdisciplinary approach of molecular modeling and computational chemistry in order to characterize the most important structure-activity features of bioactive peptides for the increasing their therapeutic index in human diseases.

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