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Two proline-rich peptides from pig (*Sus scrofa*) salivary glands generated by pre-secretory pathway underlying the action of a proteinase cleaving Pro–Ala bonds

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Abstract

The primary structures of two salivary proline-rich peptides (PRP-SP-A, M 6156.0 amu and PRP-SP-B, M 1905.0 amu), from pig (*Sus scrofa*) were determined. The PRP-SP-B peptide, 21 residues long, overlaps with a sequence repeated 43 times in three deposited cDNAs coding for PRP proteins cloned from porcine parotid glands (Swiss-Prot codes: *Q95JC9*, *Q95JD1*, *Q95JD0*). PRP-SP-A peptide, 56 amino acid residues long, overlaps with the N-terminus repeats of *Q95JC9* and *Q95JD1* and it is phosphorylated at Ser 12 and 14. The two peptides were found both in whole saliva and in granules from pig parotid glands. The biosynthesis of the two peptides implies the action of a proteinase responsible for Pro↓Ala cleavage in the pre-secretory process.

Keywords: Proline-rich proteins; Saliva; Salivary glands; Parotid; Pig; *Sus scrofa*; Prolyl–alanyl proteinase

1. Introduction

Mammal saliva contains different specific classes of peptides and proteins. Among them, the class of peptides with abnormal elevated presence of proline residues (salivary proline-rich proteins; PRPs) usually represents more than 50% of the total whole salivary protein complex [7]. Primary structures of some human PRPs have been characterized [15,20], while those of other mammals are quite incomplete. For this reason, a comparative study is developing in our laboratories addressed towards a more complete characterization of the protein complex of mammal saliva, in the hope that structural similarities and differences observed with respect to human salivary PRPs could offer some novel information on the role of these proteins in the maintaining of oral health. This study describes the characterization of two salivary proline-rich peptides from pig (*Sus scrofa*). The principal information on structures and genetic relationships of salivary proline-rich peptides derives from human saliva [4,6,12,24]. On the basis of the ionogenic properties, salivary PRPs can be subdivided into acidic (A-PRPs), basic (B-PRPs) and basic glycosylated (G-PRPs) proline-rich proteins. Acidic PRPs seem contribute to maintain calcium homeostasis in the oral cavity [8], glycosylated PRPs act as lubricants [14], whereas the role of basic PRPs is not well understood. Probably one of their functions is to bind tannins, preventing their toxic effects at the gastro-intestinal tract [17]. Recently,

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it has been hypothesized that a not identified basic PRP exerts a protection against HIV infectivity in the oral cavity [5,22]. The number of human PRPs is not completely defined. More than 11 human basic-PRPs and more than five acidic PRP isoforms have been identified [15,20]. They derive from different genes where homologous and unequal crossing-over produce frequent length polymorphisms [7,16,18,19]. Multiple PRPs may originate from the same gene throughout allelic variations and differential splicing. However, post-translational cleavages from larger precursors have been recognized as a widespread motif in their biosynthesis, as well as for that one of other salivary peptides. Therefore, it is important to establish either the cleavage occurs during granule maturation or it is generated after salivary secretion by exogenous proteinases present in the oral cavity. In this aim, the peptides characterized in whole pig saliva were also searched with success in granule preparations of pig parotid.

2. Materials and methods

2.1. Reagents

All general chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba (Milan, Italy), Merck (Darmastad, Germany), or Sigma–Aldrich (St. Louis, MI, USA).

2.2. Sample collection and treatment

Salivary samples were collected from four anesthetized pigs (Landrace bred). Pigs were all females, about 90-days old, mean weight about 30 kg. Pre-anesthesia was performed without atropine by intramuscular administration of ketamine (15 mg/kg) and diazepam (0.1 mg/kg). Anesthesia induction was performed by inhalation mask with a gaseous mixture of O_2/isoflurane 98/2 (v/v%). Saliva secretion was stimulated by sublingual direct administration of pilocarpine and carbachol (2 and 4 mg/kg, respectively; powder). Whole saliva was collected by means of plastic aspirators. It was immediately diluted (1:1 v/v) with H_2O/TFA 0.2% in ice bath and centrifuged at 10,000 × *g* for 5 min. The precipitate was discharged and the acidic solution was either used immediately for chromatographic separations or stored at −80 °C. The acidic treatment of salivary samples caused the precipitation of high molecular weight proteins and partly inhibited salivary proteinases, preserving sample protein composition for the following analysis. Experiments were carried out in accordance with the EU (86/609/EEC) ethical guidelines.

2.3. Salivary gland granules preparation

Granules were isolated according to the procedure of Silva and colleagues [23] with some modifications. The glands were homogenized by Ultra-Turrax T25 as a 5% (w/v) suspension in the following medium: sucrose 340 mM, EDTA 0.5 mM, HEPES buffer 10 mM, pH of 7.4. In order to remove fibrous connective tissue and insoluble particles, homogenate was filtered through four layers of clean cheesecloth in the homogenizing medium and then centrifuged at 500 × *g* for 30′. The soluble solution was submitted to further centrifugation at 2500 × *g* for 30′ and the pellet corresponding to the crude fraction of secretory granules was suspended in 700 μl of 0.2% formic acid. Solution was centrifuged at 800 × *g* for 30′ and $CHCl_3$ was added to the solution (1:1 v/v). The separated aqueous phase was newly centrifuged and the solution was stored at −20 °C.

2.4. Apparatus

The HPLC apparatus was a System Gold HPLC, Beckman Coulter (Fullerton, CA, USA) using a LIChrosorb RP-18 column (7 μm, 4.6 mm × 250 mm). The HPLC-ESI-MS apparatus was a ThermoFinnigan (San Jose, CA, USA) Surveyor HPLC connected by a T splitter to a PDA diode-array detector to Xcalibur LCQ deca-Xplus mass spectrometer. The mass spectrometer was equipped with an electrospray ion (ESI) source. The resolution of the mass spectrometer was in the range of 0.4 FWHM. The chromatographic column was a Vydac (Hesperia, CA, USA) C_8 column, with 5 μm particle diameter (column dimension 150 mm × 2.1 mm). Peptide sequence was performed with a Procise 610A Protein Sequencer (Applied Biosystems, Foster City, CA, USA).

2.5. Protein isolation and identification

Preparative separations were performed in a linear gradient HPLC system, using a LiChrosorb RP-18 (7 μm, 4.6 mm × 250 mm) column. Eluting peptides (eluant A: 0.2% TFA aqueous solution; eluant B: acetonitrile/solution A 80:20 v/v) were monitored at 214 nm wavelength. The single collected peaks were further analyzed by HPLC-ESI-IT mass spectrometry. The following solutions were utilized for the reversed-phase chromatography: (eluant A) 0.056% aqueous TFA and (eluant B) 0.050% TFA in acetonitrile–water 80/20 (v/v). The applied gradient was linear from 0 to 55% in 40 min, at a flow rate of 0.30 ml/min. A T splitter addressed a flow-rate of about 0.20 ml/min towards the diode array detector and a flow-rate of about 0.10 ml/min towards the ESI source. The diode array detector was usually set in the wavelength range of 214–276 nm. Mass spectra were collected every 3 ms in the positive ion mode. MS spray voltage was 4.50 kV and the capillary temperature was 220 °C. De-convolution of averaged mass spectra were automatically performed by software provided with the instrument.

2.6. Trypsin digestion and analysis of tryptic digest by HPLC-ESI MS-MS

The lyophilized peptide (1 mg) was dissolved in 1.9 ml of distilled water and the pH was adjusted to 8.8 by addition of NaOH 0.1 M. Ammonium hydrogencarbonate (4.0 mg)

and 50 μl of trypsin dissolved in HCl 1 mM (2 mg/ml) were added. Cleavage was performed for 6 h at 37 °C. The digest was brought to pH 2.0 by HCl 1 M, lyophilized and stored at −20° C. The HPLC-ESI MS-MS analysis of the tryptic digest was performed according to the procedure described in the previous section.

2.7. Data analysis

Mass values obtained by HPLC-ESI-MS analyses were compared with averaged theoretical values available at SWISS-PROT (http//www.expasy.ch) and EMBL (http//www.embl-heidelberg.de) data bank. Tandem-MS data were compared with the theoretical fragmentation patterns obtainable by Protein Prospector (http//prospector.ucsf.edu/) program.

3. Results

Salivary samples of pig were collected from anesthetized animals treated with carbachol and pilocarpine as stimulants in order to increase saliva secretion. A study on the effect of stimulants was not performed. Pig saliva was mixed as obtained in a 1:1 (v/v) ratio with an aqueous solution of TFA 0.2% (v/v) (see Section 2). The precipitate was discharged and the acidic soluble solution was submitted to RP-HPLC analysis at 214 nm and RP-HPLC-ESI-MS (Fig. 1a) showing a complex chromatographic profile. Even though the profiles obtained analyzing whole salivary samples obtained from different animals was roughly similar, minor differences were often observed, probably linked either to individual variations or to different gland contributions. One of the components of the salivary protein complex, having M (average) 6156.0 ± 0.8 amu was named PRP-SP-A (Proline-Rich-Peptide-Salivary-Pig-A) and another one having M (average) 1904.0 ± 0.4 amu was named PRP-SP-B (Fig. 1b). The mass values were obtained by de-convolution of different ESI spectra recorded in the central part of the chromatographic peaks. PRP-SP-A and PRP-SP-B were collected by preparative HPLC separation and submitted, without further purification, to automated Edman degradation. The sequence of PRP-SP-B was the following:

1	11	21
APPGARPPPG	PPPPGPPPPG	P

The sequence determined exactly matches the experimental mass determined by ESI-MS experiments (M exp. 1904.0 ± 0.4 amu; M theor. 1904.2 amu). The sequence of PRP-SP-B peptide was confirmed by MS-MS experiments (Fig. 2).

Automated sequencing of PRP-SP-A peptide (M 6156.0 ± 0.8 amu) allowed to obtain primary structure information up to the 27th residue with two undetermined residues at 12 and 14 positions:

- RSPFFDLEDA NXNXAEKFLR PPPGGGP. . ..

A similarity search carried out with BLAST algorithm software at the Expasy-EMBL/GenBank database, revealed that the sequence of PRP-SP-B and the partial sequence of PRP-SP-A overlap several regions of three different cDNA sequences (*Q95JD1*, *Q95JD0*, *Q95JC9*) corresponding to bigger hypothetical basic PRP proteins. PRP-SP-B sequence match with 43 contiguous and non-contiguous repeats of the three cDNA sequences (11 on *Q95JD1*, 10 on *Q95JD0*, 22 on *Q95JC9*).

The molecular mass of PRP-SP-A peptide exactly corresponds to the N-terminal fragment 1–56 of the two cDNA sequences *Q95JD1* and *Q95JD0*, assuming that the two undetermined residues at 12 and 14 positions (serines from the cDNA sequence) were phosphorylated (M av. exp = 6156.0 ± 0.8 amu; M av. theor. 6156.4 amu). On this basis, the complete sequence of PRP-SP-A is the following:

1	11	21	31	41	51
RSPFFDLEDA	NSNSAEKFLR	PPPGGGPPRP	PPPEESQGEG	HQKRPRPPGD	GPEQGP

where *S* symbol corresponds to phosphorylated serines. This sequence was confirmed after tryptic digestion of purified PRP-SP-A peptide and MS-MS experiments (Fig. 3). The three fragments were: 1–17 which mass value corresponds to the diphosphorylated peptide ($[M + H]^+ = 2088.0 \pm 0.5$ amu), 18–43 ($[M + H]^+ = 2747.0 \pm 0.6$ amu) and 44–56 ($[M + H]^+ = 1360.5 \pm 0.4$ amu).

In order to verify that PRP-SP-A and PRP-SP-B peptides were not originated by post-secretion proteolysis in pig saliva, granule preparations from pig parotid glands, where analyzed. The HPLC-ESI-MS profile of the protein content of parotid granules showed the presence of the two peptides in a quantity comparable to that observed in whole saliva (Fig. 4). The peptide sequences will appear in the Swiss-Prot and TrEMBL knowledgebase under the accession numbers P85405 and P84506 for PRP-SP-A and PRP-SP-B, respectively.

4. Discussion

The results of this study suggest that PRP-SP-A and PRP-SP-B peptides are generated from higher molecular weight precursors before or during the maturation of granules of parotid glands. The three cDNA sequences of the precursor protein, deposited in Swiss-Prot data bank, account for proteins of 550 (*Q95JD1*), 495 (*Q95JD0*) and 660 (*Q95JC9*) amino acid residues, respectively. Interestingly, in the three cDNA sequences multiple repeats could be responsible for

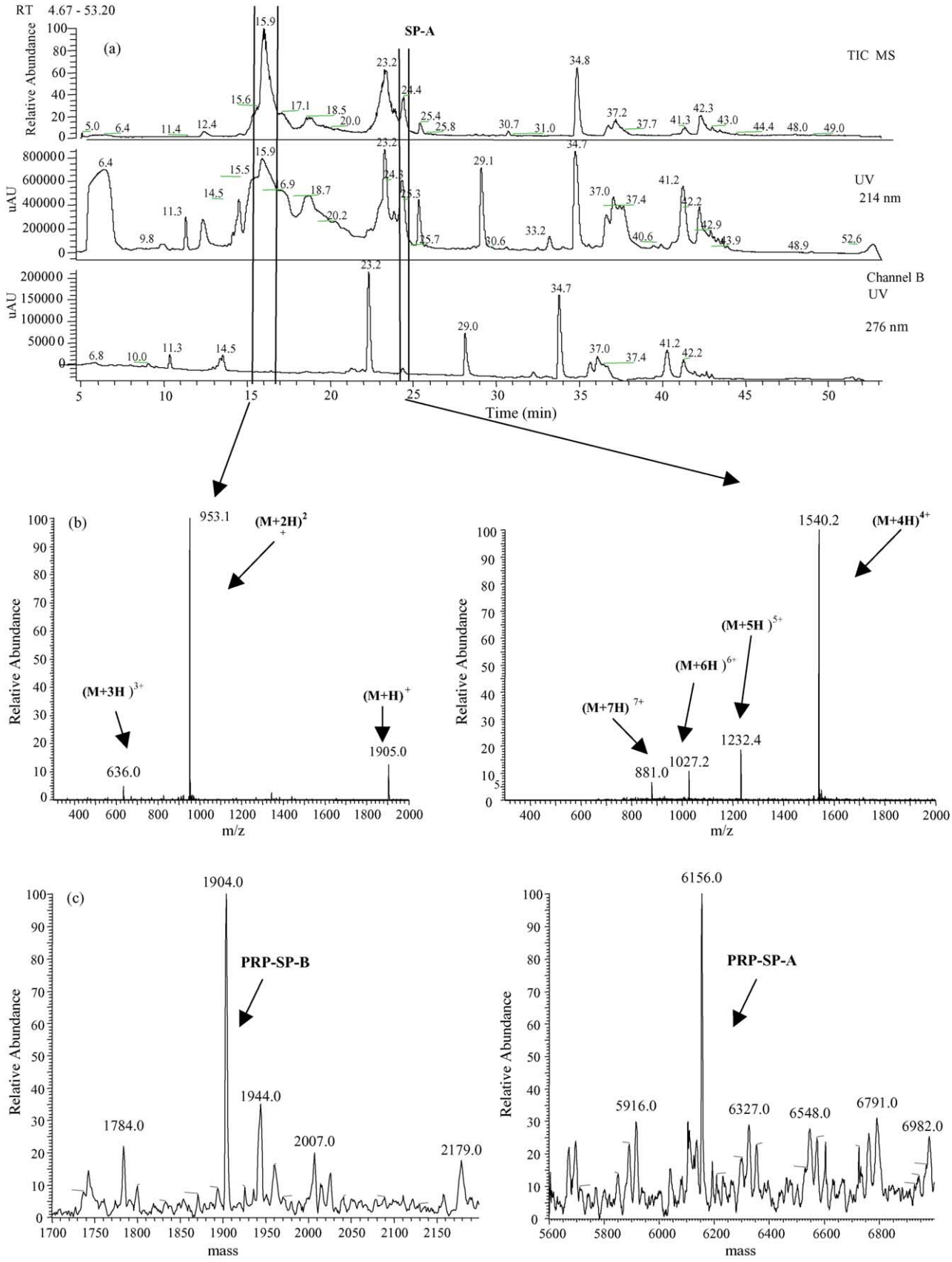

Fig. 1. Panel a: ionic current and UV (214 and 276 nm) chromatograms of pig saliva preparation. Panel b: ESI-IT mass signals of PRP-SP-A and PRP-SP-B peptides. Their de-convolution and relative molecular mass is reported in Panel c.

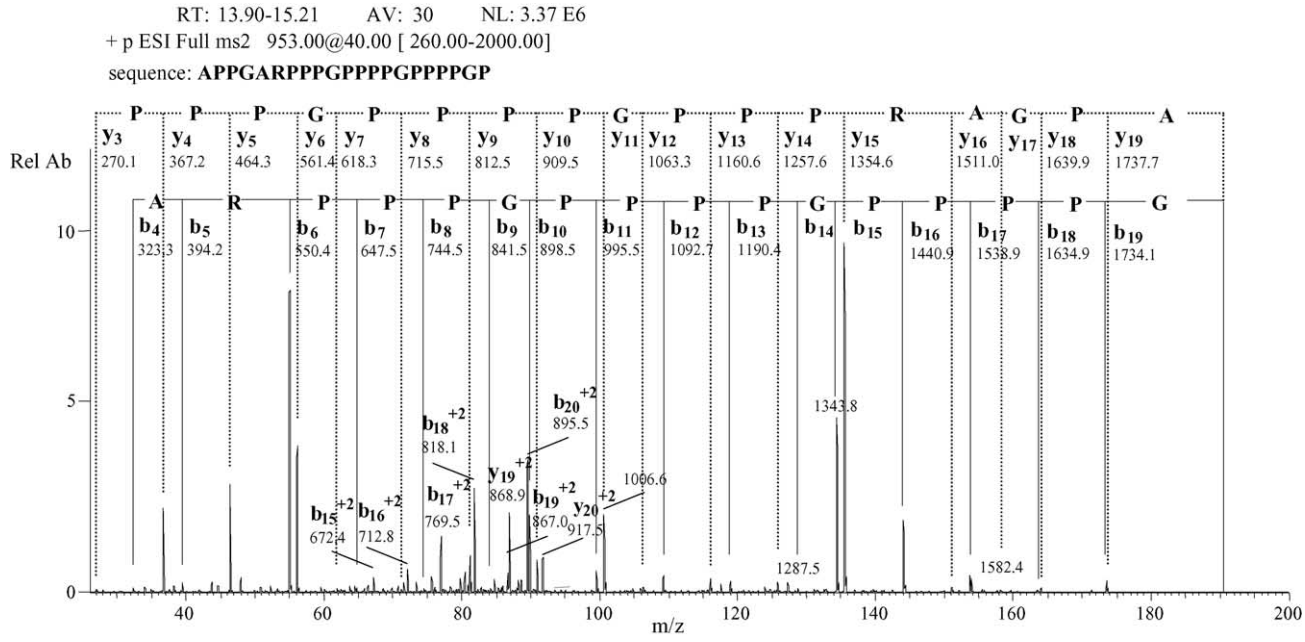

Fig. 2. MS/MS experiment of PRP-SP-B peptide performed on the doubled charged ion with a *m*/*z* value of 953.0. The analysis of the fragmentation pattern allowed recognize the y and b (mono- and di-charged) fragmentation series.

the generation of the smaller peptides: the two N-terminal 1–56 sequences of the *Q95JD1* and *Q95JD0* for PRP-SP-A peptide and 43 contiguous and non-contiguous repeats for PRP-SP-B peptide. Table 1 reports the overlapping of PRP-SP-A and PRP-SP-B with the *Q95JD0* cDNA, in example.

Moreover, MS experiments showed that the PRP-SP-A peptide is diphosphorylated at Ser 12 and Ser 14. It is worthwhile to outline that NetPhos 2 program provided high scores (0.95) for Ser 2 and 36 residues as potential sites of phosphorylation, whereas the two effectively phosphorylated residues (Ser 12 and Ser 14) obtained a lower score (about 0.45). Similar result was observed in human PRPs, which are phosphorylated by uncommon kinases that do not recognize the consensus sequences used in the algorithm of NetPhos 2. Recently, Brunati and colleagues [9] reported that in human salivary PRP-1 the phosphorylation is probably under the action of a Golgi-CK-like kinase able to recognize either SXE/S(phos) or $SXQXX(D/E)_3$ consensus sequences. A similar pig kinase could be responsible for the phosphorylation both at Ser 12 and 14. Ser 14 should be the first serine phosphorylated according to the flanking SXE sequence and Ser 12 the second one, according to the SXS(phos) flanking consensus sequence generated by the first phosphorylation.

The process of PRP-SP-A and PRP-SP-B production in some way resembles the pathway observed for human PRPs. These proteins are generated by a complex pathway involving the cleavage of the primary transcription products by a proteolytic process [19]. Interestingly, the identical leader sequence of 16 amino residues of the three cDNA (MLPILLSVAL LALSSA) is quite coincident with the leader sequence of human acidic PRPs (*P02810*) and with those reported for human basic IB-6 PRP (*P04280*) and for human basic glycosylated PO peptide (*P10163*). Similarly to the cleavage of human acidic PRP-1 (150 residues), which generates an acidic peptide named PRP-3 (106 residues) and a basic PC peptide (43 residues), the PRP-SP-A peptide is greater and acidic (pI th. 4.6 non-phosphorylated; 4.0 diphosphorylated) and PRP-SP-B peptide is smaller and basic (pI th. 9.1). The acidic residues of PRP-SP-A are more confined towards the N-terminus of the sequence and the basic ones towards the C-terminus, even though they are more dispersed in the sequence with respect to human PRP-3. SP-A peptide shares part of its sequence with acidic proline-rich proteins, in particular from human and rat (Table 2), and the similarity pertains to the portion between acidic and basic portion of the proteins.

PRP-SP-B peptide shares its sequence with *Capreolus capreolus* and *Mus musculus* parotid salivary proline-rich proteins, having a common sequence GPPP and its palindromic analogues, as shown in Table 3. It should be noticed the similarity between *Capreolus capreolus* and pig sequences. On the contrary, the pig sequence has a low similarity with other known mammals PRPs. The most relevant difference with respect to the known sequence of human is the complete lacking of glutamine residues between the polyproline repeats. Because glutamine residues could play a relevant role in polyphenol binding activities, as demonstrated for human and rats PRPs [17], the structural difference of PRP-SP-B suggests a different role of this peptide in pig oral cavity. Its structural features suggest consider this compound as a possible member of the “proline-rich peptide” family, whose components show interesting antimicrobial activity [1]. This defensive role would justify the

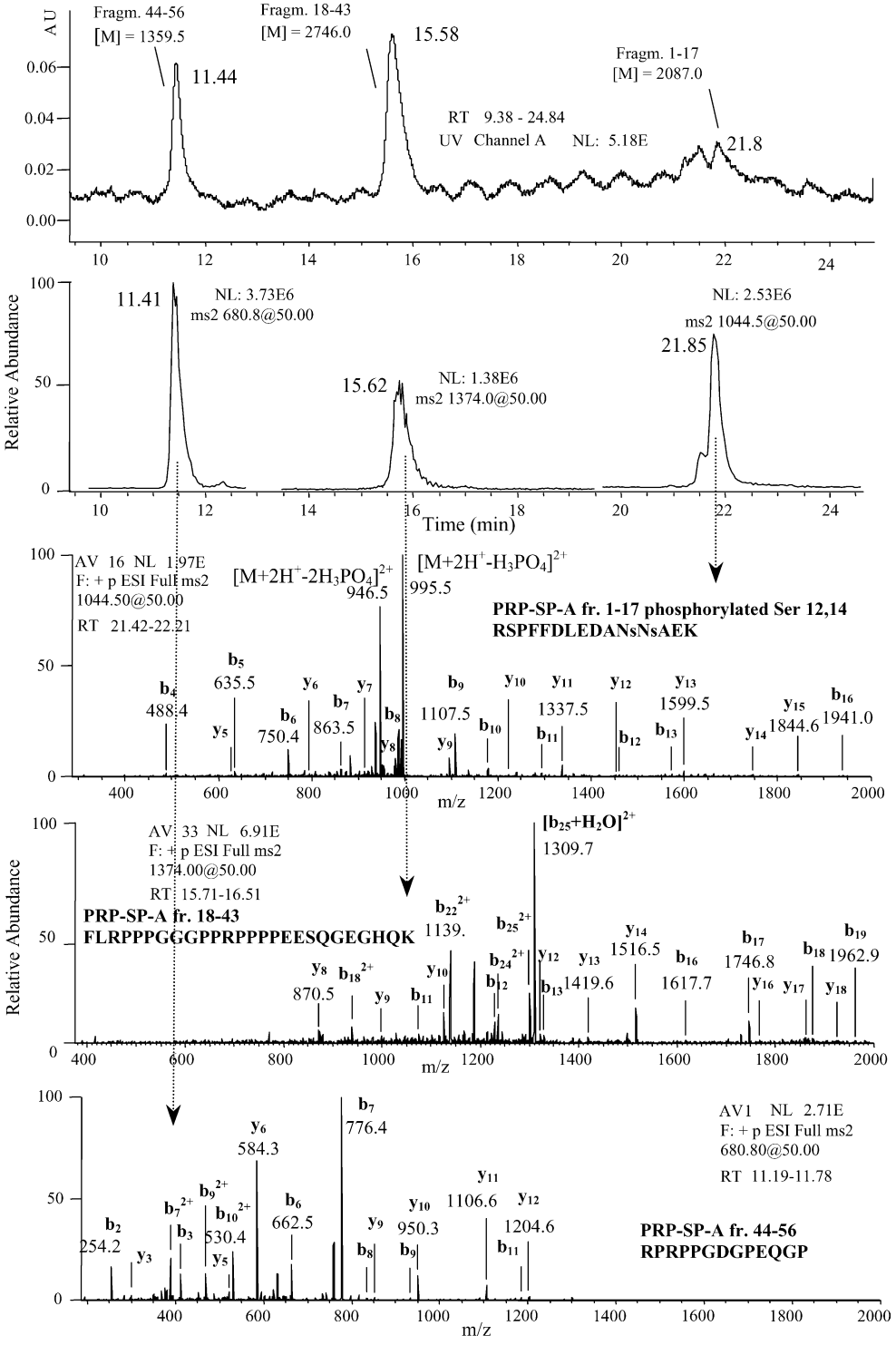

Fig. 3. (a) TIC and (b) selected ion monitoring (SIM) profiles of a MS-ion trap experiment performed for the search of tryptic fragments of PRP-SP-A. SIM profiles show the sum of ionic current relative to: (c) di- and tri-charged ions of 2088.0 (1–17 fragment); (d) di- and tri-charged ions of 2747.1 (18-43 fragment); (e) mono- and di-charged ions of 1360.5 (44–56 fragment), respectively.

presence of multiple repeats in the cDNA sequences and the high production of this peptide in the pig oral cavity.

However, the cleavages observed for the generation of many human salivary peptides/proteins suggested the widespread action of a proprotein convertase of the kexin-subtilisin family which recognize a RXXR↓ consensus sequence [11]. The cleavage is often followed by the removing of the C-terminal Arg, by the action of a carboxypeptidase probably pertaining to CPZ family [21]. The cleavage process recognized in the secretion of human salivary peptides is

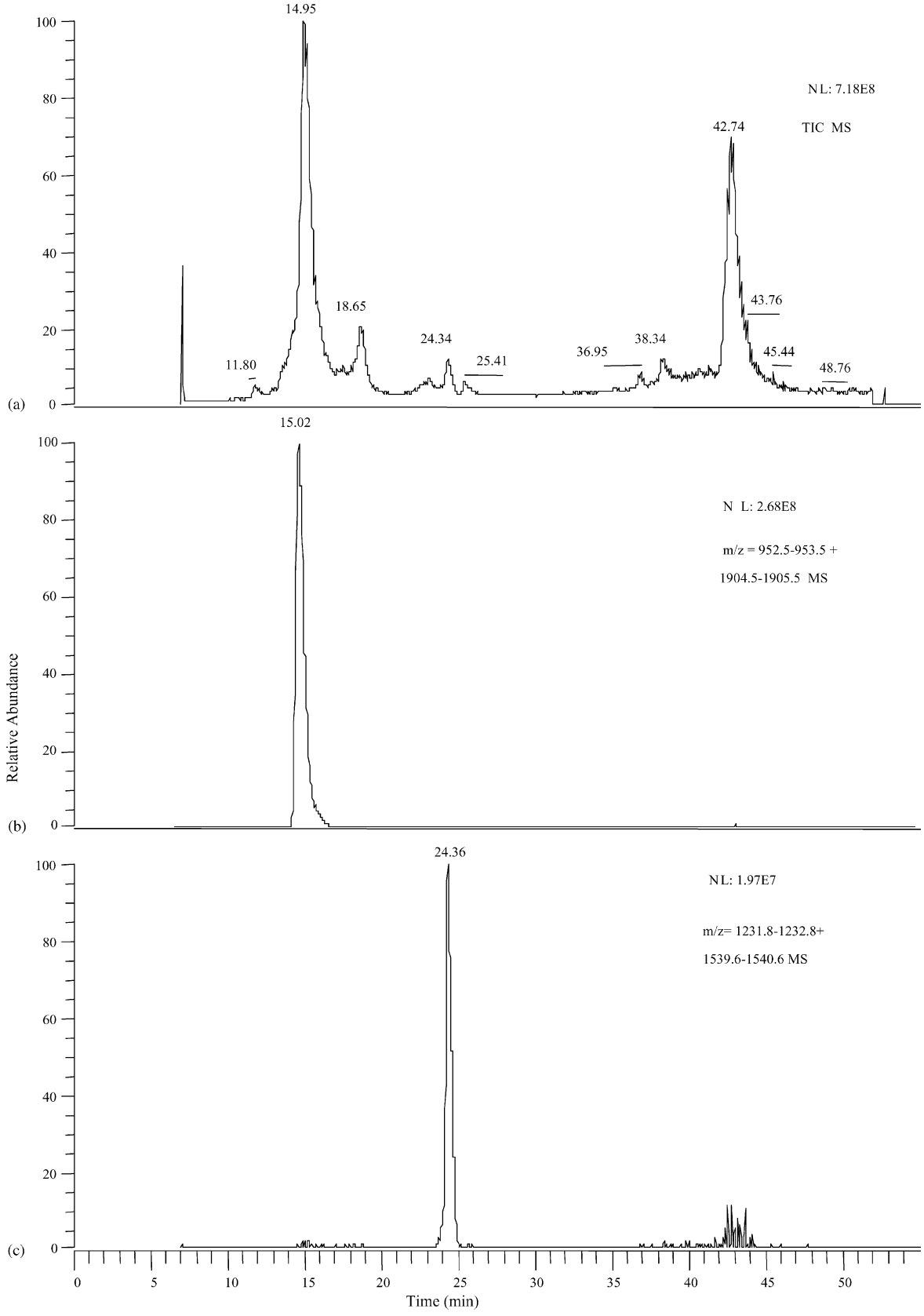

Fig. 4. (a) TIC and selected ion monitoring (SIM) profiles of MS experiments performed for the search of PRP-SP-A and PRP-SP-B peptides in granules of pig parotid glands. SIM profiles show the sum of ionic current relative to: (b) mono- and di-charged ions of 1904.0 (PRP-SP-B); (c) quadri- and penta-charged ions of 6156.0 (PRP-SP-A).

Table 1
Transcript of *Q95JD0* cDNA deposited parotid pig sequences and identification of the portions coding PRP-SP-A and PRP-SP-B peptides

```
1    gagaacaacc gcagcacttc ctccaag-atg-ctg-ccg-atc-ctg-ctc-tca-gtg-gcc-ttg-ctg-
      ---------------------------- Met Leu Pro Ile Leu Leu Ser Val Ala Leu Leu
61     gcc-ctg-agc-tca-gct-cgg-agc-ccg-ttt-ttt-gat-tta-gag-gat-gca-aac-tca-aac-
       Ala Leu Ser Ser Ala Arg Ser Pro Phe Phe Asp Leu Glu Asp Ala Asn Ser Asn
115    tcc-gct-gag-aag-ttt-ctg-aga-cca-cct-cct-gga-ggc-gga-cca-ccc-agg-ccc-cct-
       Ser Ala Glu Lys Phe Leu Arg Pro Pro Pro Gly Gly Gly Pro Pro Arg Pro Pro
169    cct-cct-gag-gaa-agc-caa-ggt-gag-gga-cat-cag-aag-aga-cca-cga-cca-cct-ggt-
       Pro Pro Glu Glu Ser Gln Gly Glu Gly His Gln Lys Arg Pro Arg Pro Pro Gly
223    gac-gga-ccg-gag-cag-gga-cca-gcc-cca-cct-ggt-gcc-aga-ccc-ccg-ccc-gga-ccg-
       Asp Gly Pro Glu Gln Gly Pro♦Ala Pro Pro Gly Ala Arg Pro Pro Pro Gly Pro
277    ccc-cca-ccc-gga-ccg-ccg-cca-ccc-gga-ccc-gcc-cca-cct-ggt-gcc-aga-ccc-ccg-
       Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro Pro Gly Ala Arg Pro Pro
331    ccc-gga-ccg-ccc-cca-ccc-gga-cca-ccg-cca-ccc-gga-cca-gcc-cca-cct-ggt-gcc-
       Pro Gly Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro Pro Gly Ala
385    aga-cca-ccg-ccc-gga-ccg-ccc-cca-ccc-gga-cca-ccc-cca-ccg-gga-cca-gcc-cca-
       Arg Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro
439    cct-ggt-gcc-aga-ccc-ccg-ccc-gga-cca-ccg-cca-ccc-gct-ggc-gga-ctc-cag-cag-
       Pro Gly Ala Arg Pro Pro Pro Gly Pro Pro Pro Pro Ala Gly Gly Leu Gln Gln
493    gga-cca-gcc-cca-tcc-cat-gtt-gga-cca-aag-aag-aaa-cca-cct-cca-ccc-ggt-gcc-
       Gly Pro Ala Pro Ser His Val Gly Pro Lys Lys Lys Pro Pro Pro Pro Gly Ala
547    gga-cac-cct-ccc-aga-cca-ccc-cca-cct-gct-aac-gaa-tcc-cag-ccg-gga-ccc-aga-
       Gly His Pro Pro Arg Pro Pro Pro Pro Ala Asn Glu Ser Gln Pro Gly Pro Arg
601    cct-ccg-ccg-gga-cca-cca-tca-cca-ccc-gct-aac-gat-agc-cag-gag-gga-tca-ccc-
       Pro Pro Pro Gly Pro Pro Ser Pro Pro Ala Asn Asp Ser Gln Glu Gly Ser Pro
655    cca-tcc-gct-gac-gga-ccc-cag-caa-gga-cca-gcc-cca-tcc-ggt-gac-aaa-cca-aag-
       Pro Ser Ala Asp Gly Pro Gln Gln Gly Pro Ala Pro Ser Gly Asp Lys Pro Lys
709    aag-aaa-cca-ccc-cca-ccc-gct-ggc-cca-ccc-cca-cca-ccc-cca-ccc-cca-ccc-gga-
       Lys Lys Pro Pro Pro Pro Ala Gly Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly
763    cca-ccg-cct-cct-gga-cca-gcc-cca-cct-ggc-gcc-aga-ccc-ccg-ccc-gga-ccg-ccc-
       Pro Pro Pro Pro Gly Pro♦Ala Pro Pro Gly Ala Arg Pro Pro Pro Gly Pro Pro
817    cca-ccc-gga-ccg-ccg-cca-ccc-gga-cca-gcc-cca-cct-ggc-gcc-aga-ccc-ccg-ccc-
       Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro Pro Gly Ala Arg Pro Pro Pro
871    ggt-ccg-ccc-cca-ccc-gga-ccg-ccg-cca-ccc-gga-cca-gcc-cca-cat-ggc-gcc-aga-
       Gly Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro His Gly Ala Arg
925    ccc-ccg-ccc-ggt-ccg-ccc-cca-ccc-gga-ccg-ccg-cca-ccc-gga-cca-gcc-cca-cct-
       Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro Pro
979    ggc-gcc-aga-ccc-ccg-ccg-gga-cct-cca-cca-cca-gga-cca-ccg-cac-ccg-gac-cca-
       Gly Ala Arg Pro Pro Pro Gly Pro Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦
1033   gcc-cca-cct-ggc-gcc-aga-ccc-ccg-ccc-gga-ccg-ccc-cca-ccc-gga-ccg-ccg-cca-
       Ala Pro Pro Gly Ala Arg Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro Pro Pro
1087   ccc-gga-cca-gcc-cca-cct-ggt-gcc-aga-ccc-ccg-ccg-gga-cct-cca-cca-cca-gga-
       Pro Gly Pro♦Ala Pro Pro Gly Ala Arg Pro Pro Pro Gly Pro Pro Pro Pro Pro Gly
1141   cca-ccg-cca-ccc-gga-cca-gcc-cca-cct-ggt-gcc-aga-ccc-ccg-ccc-gga-ccg-ccc-
       Pro Pro Pro Pro Gly Pro▼Ala Pro Pro Gly Ala Arg Pro Pro Pro Gly Pro Pro
1195   cca-ccc-gga-ccg-ccg-cca-ccc-gga-cca-gcc-cca-cct-ggt-gcc-aga-ccc-ccg-cct-
       Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro Pro Gly Ala Arg Pro Pro Pro
1249   gga-ccg-ccc-ccg-ccc-gga-ccg-ccg-cca-ccc-gga-ccc-gcc-cca-cct-ggt-gcc-aga-
       Gly Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro♦Ala Pro Pro Gly Ala Arg
1303   ccc-ctg-cct-gga-ccg-ccc-cca-ccc-gga-cca-ccg-cca-ccc-gga-ccc-gcc-cca-cct-
       Pro Leu Pro Gly Pro Pro Pro Pro Gly Pro Pro Pro Pro Gly Pro Ala Pro Pro
1357   ggt-gcc-aga-ccc-ccg-cca-cca-cca-ccc-cca-ccc-gct-gac-gaa-ccc-cag-cag-gga-
       Gly Ala Arg Pro Pro Pro Pro Pro Pro Pro Pro Pro Ala Asp Glu Pro Gln Gln Gly
1411   cca-gcc-cca-tcc-ggt-gac-aaa-cca-aag-aag-aaa-cca-ccc-cca-ccc-gct-ggc-cca-
       Pro Ala Pro Ser Gly Asp Lys Pro Lys Lys Lys Pro Pro Pro Pro Ala Gly Pro
1465   ccc-ccg-cca-cca-cca-ccc-cca-ccc-gga-att-caa-gga-cag-aaa-atg-agc-gcg-aaa-
       Pro Pro Pro Pro Pro Pro Pro Pro Gly Ile Gln Gly Gln Lys Met Ser Ala Lys
1519   aca-ccc-gtc-ctt-cgg-aga-gcc-gtg-aca-ttg-gaa-tgt-gac-ggt-tga gcttcga
       Thr Pro Val Leu Arg Arg Ala Val Thr Leu Glu Cys Asp Gly -Stop ----------
1571   ttaccaataa aatcatcttgc atccag
```

Table 2
Comparison of PRP-SP-A sequence with salivary PRPs from human and rat

N(*t*)	Sequence	*C*(*t*)	Species
20	R**PP**PG**G**GP**PRPP**—**P**PEES**Q**GE**GHQ**KR**P**R**PP**GD**G**PE**QGP**	56	*Sus scrofa* PRP-SP-A
110	G**PP**QQ**G**GH**PRPP**RGR**P**QGPP**Q**QG**GHQ**QG**PPPP**PP**G**KP**QGP**P	150	Human salivary acidic proline-rich protein (*P02810*)
6	**DLED**ANSNSAEKFL**RPPP**G**G**GP**PRPP**PPEES**Q**GE**G**H**Q**K**RP**RP**PG**DGPEQG**P**	56	*Sus scrofa* PRP-SP-A
34	**D**V**ED**SSQRPDQGPQ**RPPP**E**G**LL**PRPP**GDSGN**Q**DD**GP**Q**QR**P**PK**PG**GHHRHP**P**	83	Human proline-rich protein 4 prec (*Q16378*)
20	R**PPP**G**GGP**PR**PPPP**EES**QG**EGH**Q**KR**P**—**RPP**GD**G**PE**QGP**	56	*Sus scrofa* PRP-SP-A
115	G**PPP**Q**GGP**QG**PP**Q**P**GNP**QG**PPP**Q**GG**P**QQ**RPP**QP**G**KP**QGP**P	154	Rat acidic proline-rich protein (*P04474*)

Table 3
Comparison of PRP-SP-B sequence with salivary PRPs from parotid glands of two *Capreolus c*. sequences and *Mus musculus* sequence

N(t)$_2$	Sequence	*C*(*t*)	Species
6	-PPP**GPPP**P**GPPP**PGP-	20	*Sus scrofa* PRP-SP-B
2	-PPP**GPPP**SGPSPPGP-	16	*Capreolus capreolus* (*Q8HXT1*)
24	-PPP**GPPP**S**GPPP**P-	36	*Capreolus capreolus* (*Q8HXS4*)
145	-RPPQ**GPPP**PGGPQP-	158	*Mus musculus* (*Q622103*)

very similar to that observed in many human endocrine and exocrine secretion pathways.

The detection of PRP-SP-A and PRP-SP-B peptides as the proteolytic products of bigger proteins for the pig salivary glands suggests also the action of a proteinase exerting its action before or during granule maturation. However, this proteinase is responsible for the cleavage of Pro–Ala bonds, an unusual consensus sequence for convertases and for proline specific peptidases [13]. Interestingly, one of the few proteinases able to cleave Pro–Ala bonds is enamelysin (matrix metalloproteinase 20, MMP-20 [3]), which is secreted into the enamel matrix during enamel development and it is identified as a predominant amelogenin-processing enzyme [2]. Indeed, enamelysin-deficient mice displayed an *amelogenesis imperfecta* phenotype [10]. It could be interesting in the future to investigate if either enamelysin or other enamelysin-like proteinases are responsible for the cleavage of pig parotid PRP. Moreover, because this cleavage seems to be a principal motif in the production of mature PRP peptides in pig, the search of similar proline peptidases in the salivary secretion pathways of other mammals could be relevant.

Many other peptide and protein masses were identified in the chromatographic profile of whole pig saliva showed in Fig. 1, which can partly correspond to other salivary PRP. The characterization of some of them is a future aim of our study. However, differently from PRP-SP-A and PRP-SP-B peptide, their characterization requires further and time-consuming purification steps.

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