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Original Paper

Mass spectrometry strategies applied to the characterization of proline-rich peptides from secretory parotid granules of pig (*Sus scrofa*)

Basic proline-rich proteins (bPRPs) are a class of proteins widely present in saliva of humans and other mammals. They are synthesized as preproproteins and enzymatically cleaved into small peptides before secretion from the salivary glands. Recently, we characterized two proline-rich peptides (SP-A and SP-B) in parotid secretory granules of pig (*Sus Scrofa*) that are derived from three isoforms of a PRP proprotein (Swiss-Prot data bank: **Q95JC9-1**, **Q95JC9-2** and **Q95JC9-3**). Together the coding regions for SP-A and SP-B, which are repeated many times, account for 52–70% of the coding regions of the PRP proproteins. This study was undertaken to identify peptides encoded by unassigned regions of the PRP proproteins. RP-HPLC-ESI-IT-MS analysis of enriched granule preparations from pig parotid glands by two different analytical strategies identified ten new proline-rich peptides derived from the three proproteins. Together with the coding regions for SP-A and SP-B already identified it was possible to assign 68–75% of the proproteins coding regions. The peptide sequences indicated a number of unusual proteolytic cleavage sites suggesting the presence of unknown proprotein convertases.

Keywords: HPLC-ESI-MS / Parotid gland / Peptides / Saliva / Salivary granules

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1 Introduction

Limited proteolysis of secretory protein precursors to produce active peptides is a widespread mechanism that enables cells of secretory tissues to regulate the level of specific bioactive peptides and to generate several products from the same precursor. Secretory proteins are usually synthesized as larger preproprotein precursors, which are modified during the secretory process to generate the mature molecule. Proteins may be secreted from the cell at a rate which is limited by their time of synthesis. This constitutive secretion is considered to be the default pathway for proteins without specific retention signals. In contrast, endocrine and exocrine cells can direct a specific subset of proteins to electron-dense secretory granules, where they are stored waiting for a signal for their release. This process is called stimulated or regulated secretion [1]. Salivary protein secretion can provide a useful information about the molecular events in both the constitutive and stimulated secretory processes. Basic proline-rich proteins (bPRPs) are a class of salivary proteins generated by proteolysis from larger precursors. Even though not completely characterized, human bPRPs arise from precursors under the action of a furin-like proprotein convertase that recognizes a $R(X)_2R\downarrow$ consensus sequence [2]. Recently, studies on the salivary peptides from pig (*Sus scrofa*) whole saliva and parotid secretory granules have identified two pig parotid peptides, SP-A ($M_{average}$ 6157.6 Da) and SP-B ($M_{average}$ 1904.2 Da) [3]. They are apparently derived from post-translational fragmentation of three isoforms of a proprotein. The sequences of these proteins (Swiss-Prot data bank: **Q95JC9-1**, **Q95JC9-2** and **Q95JC9-3**) were determined by cDNA analysis [4].

The amino acid sequence of SP-A peptide corresponds to the translated 1–56 N-terminal coding region of **Q95JC9-2** and **Q95JC9-3**. This is followed by a region containing sequences identical to SP-B. The SP-B sequence is repeated ten, eleven and twenty two times in **Q95JC9-2**, **Q95JC9-3** and **Q95JC9-1**, respectively.

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Abbreviations: PRP, proline-rich protein; **TIC**, total ion current

If all the repeats in the proproteins encode secreted SP-B together with the region encoding SP-A they would account for 54, 52 and 70% of the total coding regions of **Q95JC9-2**, **Q95JC9-3** and **Q95JC9-1**, respectively.

The aim of this study was, therefore, to evaluate the presence in pig parotid granules of other peptides corresponding to the unassigned sequences in the bPRP precursors thereby allowing a better understanding of the processing of pig salivary PRPs.

2 Materials and methods

2.1 Reagents

All common chemicals and reagents were of analytical grade and were purchased from Farmitalia-Carlo Erba (Milan, Italy), Merck (Damstadt, Germany) and Sigma Aldrich (St. Louis, MO). Protease inhibitor cocktail P-2174 was purchased from Sigma Chemicals Co. (St. Louis, MO).

2.2 Extraction of the content of secretory granules

The secretory granules were isolated from parotid glands of male and female pigs (*S. scrofa*) by the method of Gerluza *et al.* [5]. The method used for extraction of proteins from the granules was a modification of a previously reported protocol [5].

The salivary gland tissue was washed with 0.9% (*m*/*v*) NaCl aqueous solution to eliminate blood and minced into small pieces using a scalpel. The tissue was suspended 5% (*m*/*v*) in 340 mM sucrose, 0.5 mM EDTA, 10 mM HEPES (pH 7.4). Protease inhibitor cocktail was added to the final suspension. The suspension was homogenized in a glass Potter Elvehjelm homogenizer with a Teflon pestle at room temperature. In order to remove fibrous connective tissue and insoluble particles, the homogenate was filtered through four layers of clean coarse gauze in the homogenizing medium and then centrifuged at $500 \times g$ for 10 min at 4°C. The supernatant was submitted to further centrifugation at $2500 \times g$ for 15 min at 4°C and the pellet was analysed by SEM (S4000 FEG SEM Hitachi, Tokyo, Japan). The pellet, characterized as crude fraction of secretory granules, was solubilized in 700 μL of 0.2% v/v TFA. The solution was centrifuged at $8000 \times g$ for 10 min and $CHCl_3$ was added to the supernatant (1:1 v/v) to remove lipids. The aqueous phase was immediately used for RP-High Pressure LC-ESI-IT-MS (RP-HPLC-ESI-IT-MS) analyses.

2.3 HPLC and MS conditions

Separation and detection of peptides was performed using a Thermo Finningan (San Josè, CA) Surveyor HPLC apparatus, connected by a T splitter to a PDA diode-array detector (DAD) and to a LCQ Deca XP Plus mass spectrometer equipped with an electrospray ion (ESI) source.

Peptides were separated on a Vydac (Hesperia, CA) chromatographic column (150×2.1 mm) with 5 μm C8 RP packing. The flow rate was 0.3 mL/min. The separation of peptides was performed by gradient elution using aqueous 0.056% v/v TFA mixture as solvent A and water – ACN 20:80 v/v 0.05% v/v TFA as solvent B. The elution gradient was linear from 0 to 55% of eluent B in 40 min. A T splitter diverted the eluent at a flow rate of about 0.2 mL/min towards the DAD and at a flow rate of about 0.1 mL/min towards the mass spectrometer source. The DAD recorded absorbance at 214 and 276 nm. The mass spectrometer operated in the positive ion mode with a capillary voltage of 4.5 kV and capillary temperature of 220°C.

MS/MS experiments were performed selecting the doubly charged ion with an isolation width of ±2 *m*/*z* values and 40 – 60% of the maximum activation amplitude.

2.4 Data analysis

The mass spectra obtained from the total ion current (TIC) chromatogram were deconvoluted using MagTran 1.0 software [6]. The experimental mass values of peptides were compared with averaged and monoisotopic theoretical values using the PeptideMass and Findpept program available from the Swiss-Prot (http://us.expasy.org/tools) data bank. The theoretical MS/MS pattern was obtained from the MS-Product program, available from the Protein Prospector site (http://prospector.ucsf.edu/).

In order to identify peptides corresponding to the unassigned cDNA regions and thereby obtain a more complete coverage of the expression of the three precursor proteins two strategies were adopted:

(i) Because SP-A and SP-B apparently arise by an unusual and characteristic Pro↓Ala cleavage a search of TIC chromatogram was undertaken for masses corresponding to peptides predicted to arise from Pro↓Ala cleavage. A MS/MS SIM experiment, centred on the *m*/*z* value of the doubly charged ion of the putative fragment, was used to determine the location in the proprotein. The experimental MS/MS fragmentation pattern was then compared to the theoretical one obtainable for each hypothesized sequence by using MS-product computer program.

(ii) An inventory of all the peptide masses present in the TIC chromatogram was evaluated for peptides that could correspond to fragments of the proproteins. Among the large number of masses identified priority was given to those without absorbance at wavelength longer than 250 nm, since pig bPRP precursors contain no tyrosine or tryptophan residues. The software FindPept was used for the search. The majority of the experimental masses were compatible with multiple locations

Table 1. Peptides derived from the three isoform proproteins identified in this study together with SP-A and SP-B sequences already identified

Name	Number of Repeats in PRP **Q95JC9-3**	Number of Repeats in PRP **Q95JC9-2**	Number of Repeats in PRP **Q95JC9-1**	M_r Exp	M_r Theor	Peptide sequence
PRP-SP-A	1	1	0	6156.9	6156.5	RSPFFDLEDA^{10}NsNsAEKFLR^{20} PPGGGPPRP^{30} PPPEESQGEG^{40}HQKRPRPPGD^{50}GPEQGP
PRP-SP-B	11	10	22	1904.1	1904.2	APPGARPPPG^{10}PPPPGPPPPG^{20}P
PRP-SP-C	1	1	1	880.9	881.0	RSPFFDL
PRP-SP-D	11	10	22	1511.1	1510.7	RPPPGPPPPGP^{10}PPPGP
PRP-SP-E	1	1	0	2733.3	2733.3	DKPKKKPPPP^{10}AGPPPPPPPP^{20}PGPPPPGP
PRP-SP-F	1	1	0	1920.3	1920.2	APPGARPLPG^{10}PPPPGPPPPG^{20}P
PRP-SP-G	1	1	0	2166.4	2166.4	APPGARPPPP^{10}PPPPADQPQQ^{20}GP
PRP-SP-H	1	1	0	2013.3	2012.3	APPGARPPPP^{10}PPPPADQPQQ^{20}
PRP-SP-I	6	6	17	3790.0	3790.0	APPGARPPPG^{10}PPPPGPPPPG^{20} PAPPGARPPP^{30}GPPPPGPPPP^{40}GP
PRP-SP-L	1	1	0	1860.1	1859.1	APPGARPPPGP^{10}PPPAGGLQQ^{20}
PRP-SP-M	1	0	1	1651.9	1652.9	APPGARPPPG^{10}PPPPPPGP
PRP-SP-N	1	0	1	1222.7	1223.4	SPPRPPPGPPP^{10}PQ

Note: s, phosphorylated serine; Theor, theoretical; Exp, experimental.

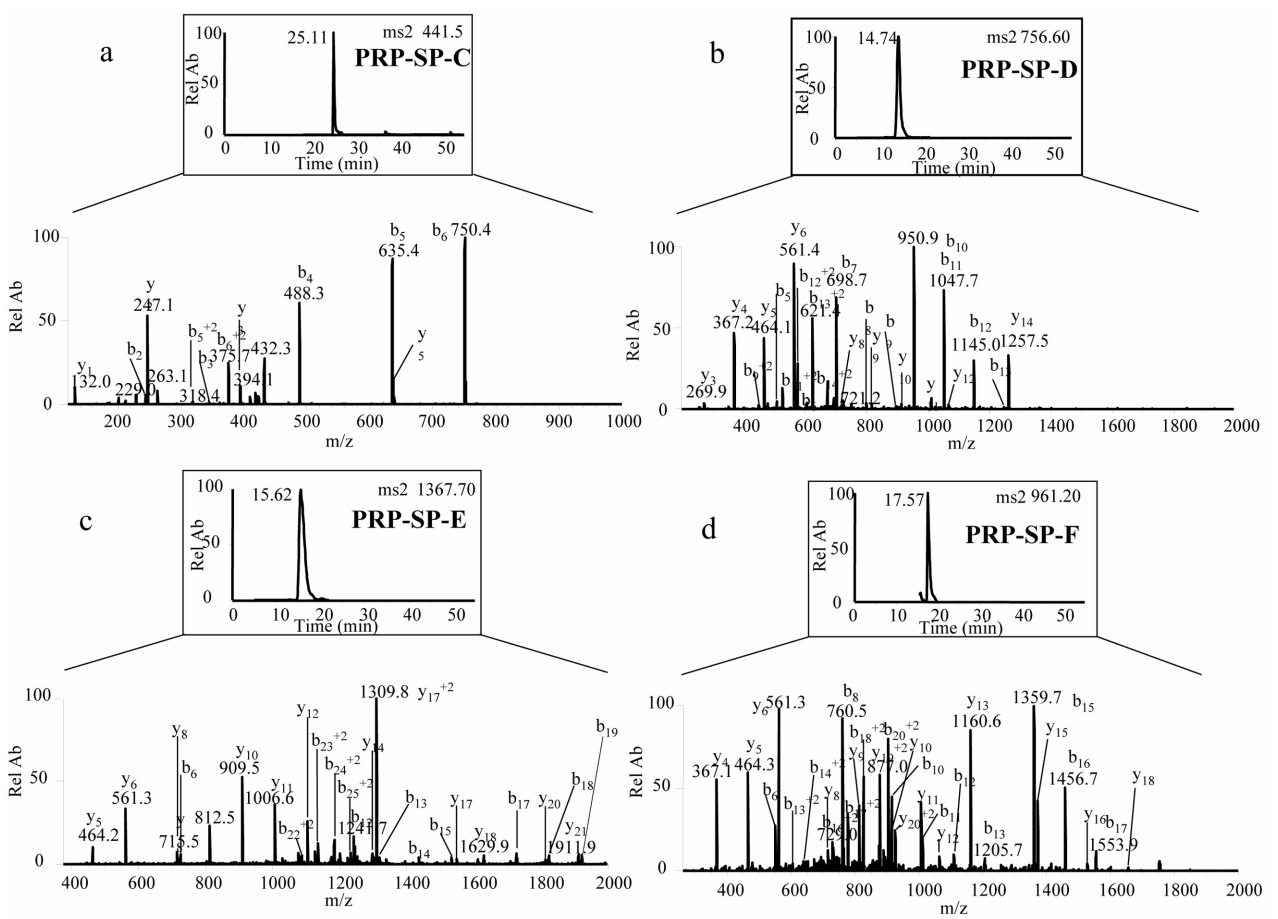


Figure 1. MS/MS spectra obtained from the fragmentation of the doubly charged ions of PRP-SP-C (a), PRP-SP-D (b), PRP-SP-E (c) and PRP-SP-F (d). In the upper part of each panel is reported the peak ion current relative to the doubly charged ion of the peptide; in the bottom part is reported the fragmentation spectrum indicating the principal ions of the *b* and *y* series.

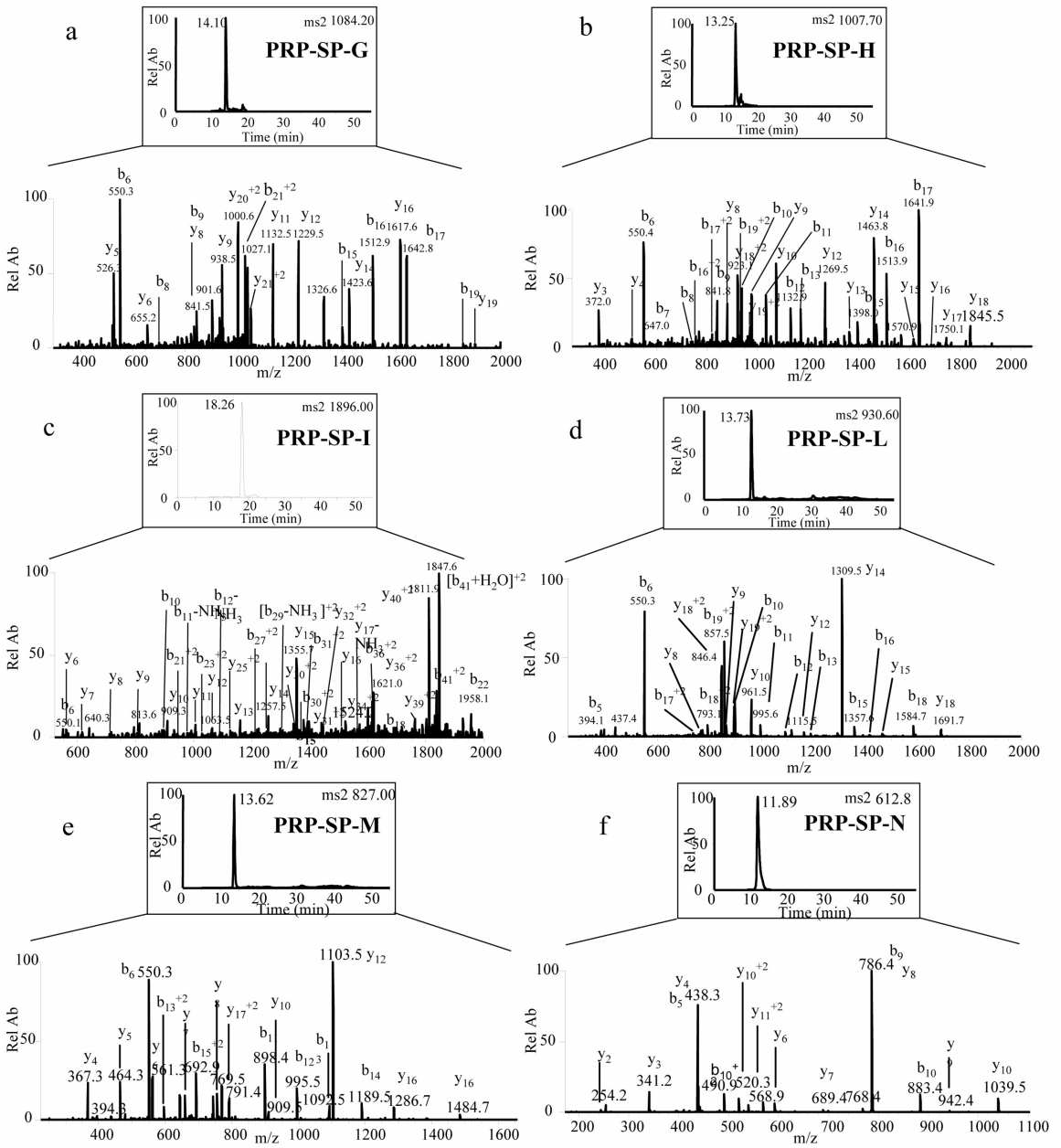
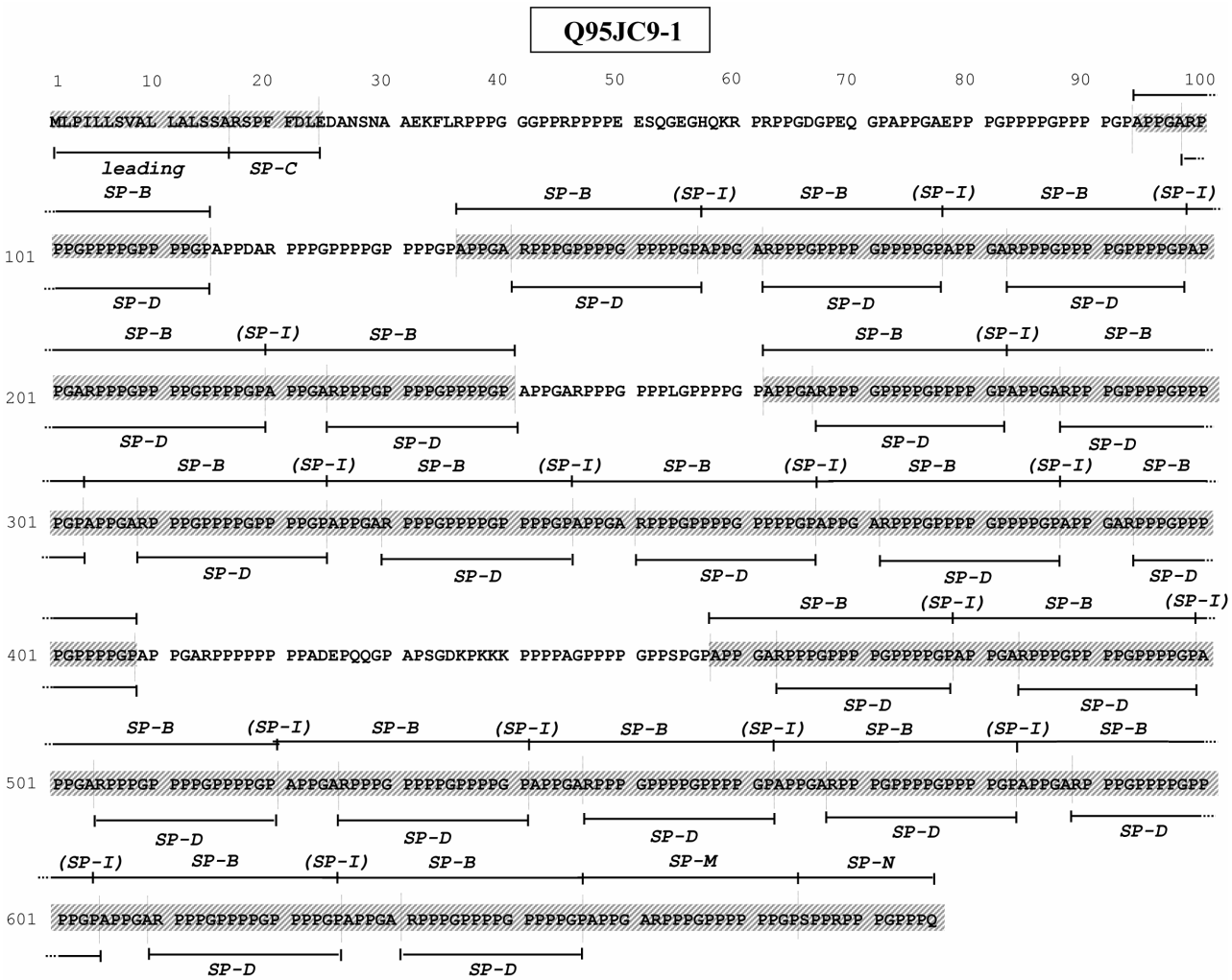


Figure 2. MS/MS spectra obtained from the fragmentation of the doubly charged ions of PRP-SP-G (a), PRP-SP-H (b), PRP-SP-I (c), PRP-SP-L (d), PRP-SP-M (e) and PRP-SP-N (f), respectively. In the upper part of each panel is reported the peak ion current relative to the doubly charged ion of the peptide; in the bottom part is reported the fragmentation spectrum indicating the principal ions of the *b* and y series.

in the PRP precursors. To solve the ambiguity MS/MS experiments were performed and analysed as reported in point (i).

3 Results

Secretory granules, analysed by SEM, appeared as an almost homogeneous preparation. Using the strategies described we were able to identify in the acidic extract of secretory granules ten new peptides derived from the three isoforms of pig basic proline-rich proprotein (Table 1). One of them (SP-I) is double the size of the previously identified SP-B and contains a tandem repeat of the SP-B sequence. Another peptide (SP-D) is a C-terminal fragment of SP-B. Peptides SP-M and SP-N together account for the sequence of the parotid hormone (30 residues peptide) described by Zhang *et al.* [4]. The other six peptides have not been described before and correspond to unassigned coding regions of the proproteins. The MS/



Fragments searched and not foumd : 17-60; 17-72; 17-93; 115-135; 241-261; 408-422; 408-430; 408-434; 431-457; 445-457; 423-430; 423-444; 431-444; 408-457.

Figure 3. Amino acid sequence of basic proline-rich proprotein isoform **Q95JC9-1**. The regions coding for the identified peptides are represented by bar lines with the indication of the peptide's name.

MS experiments leading to identification of the peptides in Table 1 are shown in Figs. 1 and 2. With this additional information it is now possible to assign 68% of **Q95JC9-2**, 70% of **Q95JC9-3** and 75% of **Q95JC9-1** coding regions. Peptides corresponding to the remaining coding regions of the proproteins were not recognized in the enriched preparations of pig salivary secretory granules. The potential coding regions that can generate the different peptides are reported in Figs. 3–5, where they are represented by a continuous line.

The peptides are generated by a number of unusual cleavages: the principal cleavage occurs at Pro–Ala bonds and the others at Pro–Ser, Glu–Asp, Ala–Arg, Gln–Gly and Gly–Asp bonds.

Among the six newly discovered peptides two are related: SP-H corresponds to SP-G peptide missing the C-terminal Gly and Pro residues. The amino acid sequences of some fragment peptides are similar to each other. In particular SP-F peptide differs from SP-B only in the substitution of a proline by leucine at position 8; moreover the N-terminal sequences of SP-G, SP-H and SP-L peptides are the same as SP-B.

The amino acid sequence of SP-E peptide has some unusual features including a tetra lysine sequence and nine consecutive proline residues suggesting the presence of a polyproline helix.

4 Discussion

Detection in pig parotid glands of the peptides reported in Table 1 imply the cleavages of Pro–Ala, Glu–Asp, Ala–Arg, Gln–Gly and Gly–Asp bonds of the proproteins. This cleavage may derive from proprotein conver-

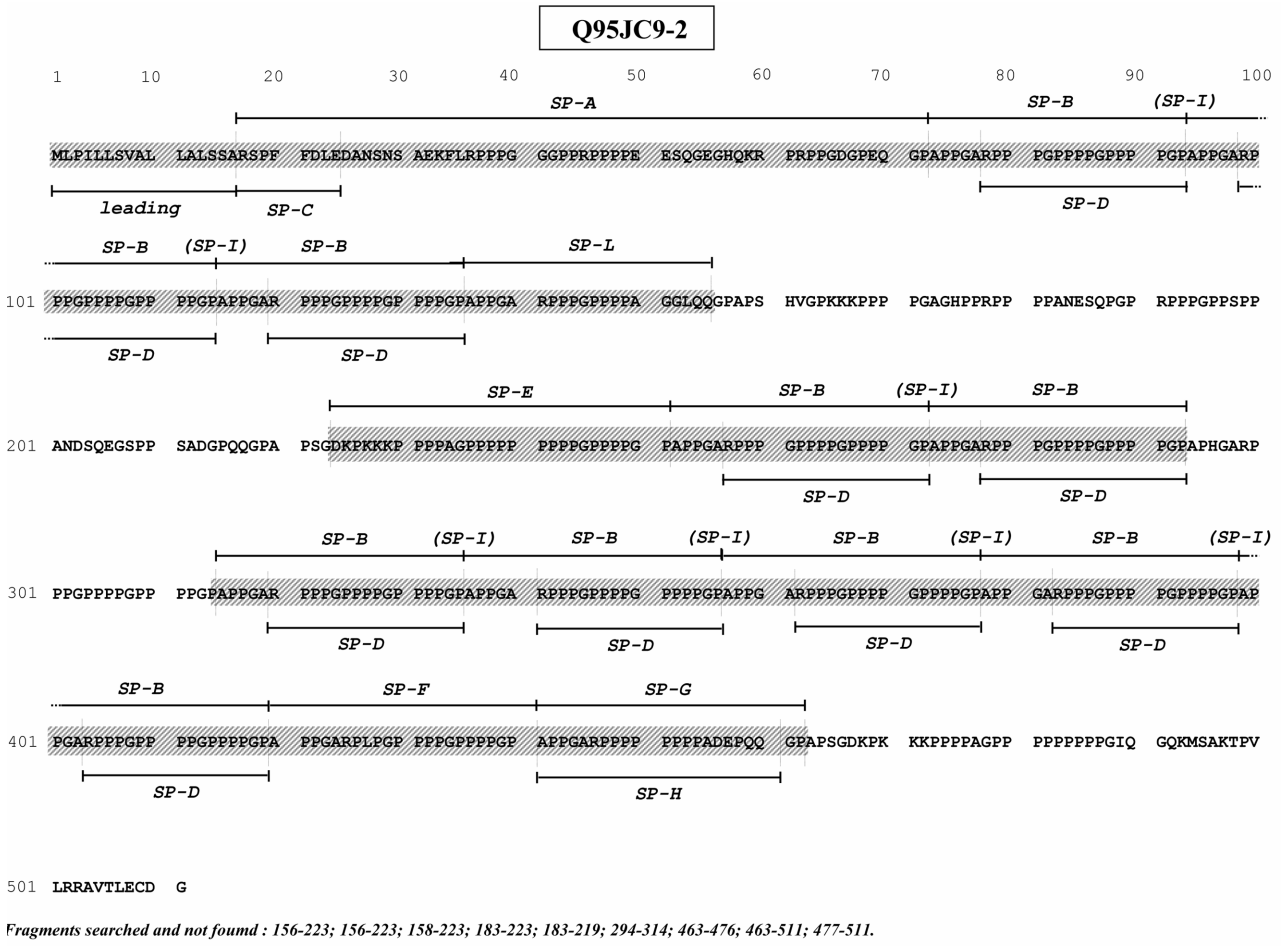


Figure 4. Amino acid sequence of basic proline-rich proprotein isoform **Q95JC9-2**. The regions coding for the identified peptides are represented by bar lines with the indication of the peptide's name.

tase(s) with different specificity from that ones acting in human glands [2] and further studies will be necessary to investigate on the proteases involved. A search of the Merops database (http://merops.sanger.ac.uk/) found that the peptidase enamelysin, acting on the protein amelogenin in tooth formation and probably responsible for the processing of all enamel proteins [7, 8], can cleave Pro↓Ala bonds *in vitro* and it could be, therefore, a potential candidate.

Zhang *et al.* [4] have shown that two of the bPRP precursor proteins (**Q95JC9-1** and **Q95JC9-3**) encode a parotid hormone in the 30 residues C-terminal region. This peptide was not present in our enriched preparations of salivary secretory granules. However, we detected two peptides, named SP-M and SP-N, corresponding to two fragments generated by a Pro↓Ser cleavage that completely cover the sequence of parotid hormone.

On the basis of the experimental procedure (use of proteinase inhibitors), SP-M and SP-N are not likely an artifact of the preparation, but they should be generated during the secretion process. Moreover, the detection of SP-M and SP-N in both male and female pigs ensures that their formation is not sex related.

Endocrine secretion of different peptides from the same protein has been already described for peptides deriving from the SMR1 protein of the submaxillary gland of rat [9].

The peptides identified in this study belong to the family of salivary bPRPs although their sequences show noticeable differences in term of proline-rich repeat length from bPRPs of other mammals. It is conceivable that these differences may also play a role in the bacterial colonization of the mouth. Besides the best-known bPRPs of human, several bPRPs of rabbit [10, 11], rat [12 – 14], hamster [15] and monkey [16, 17] have been characterized.

The role of these proteins is not completely clear, although they could have antiviral effects [18] and are probably involved in the protection against toxic effects of tannins in the gastrointestinal tract [19], this last fea-

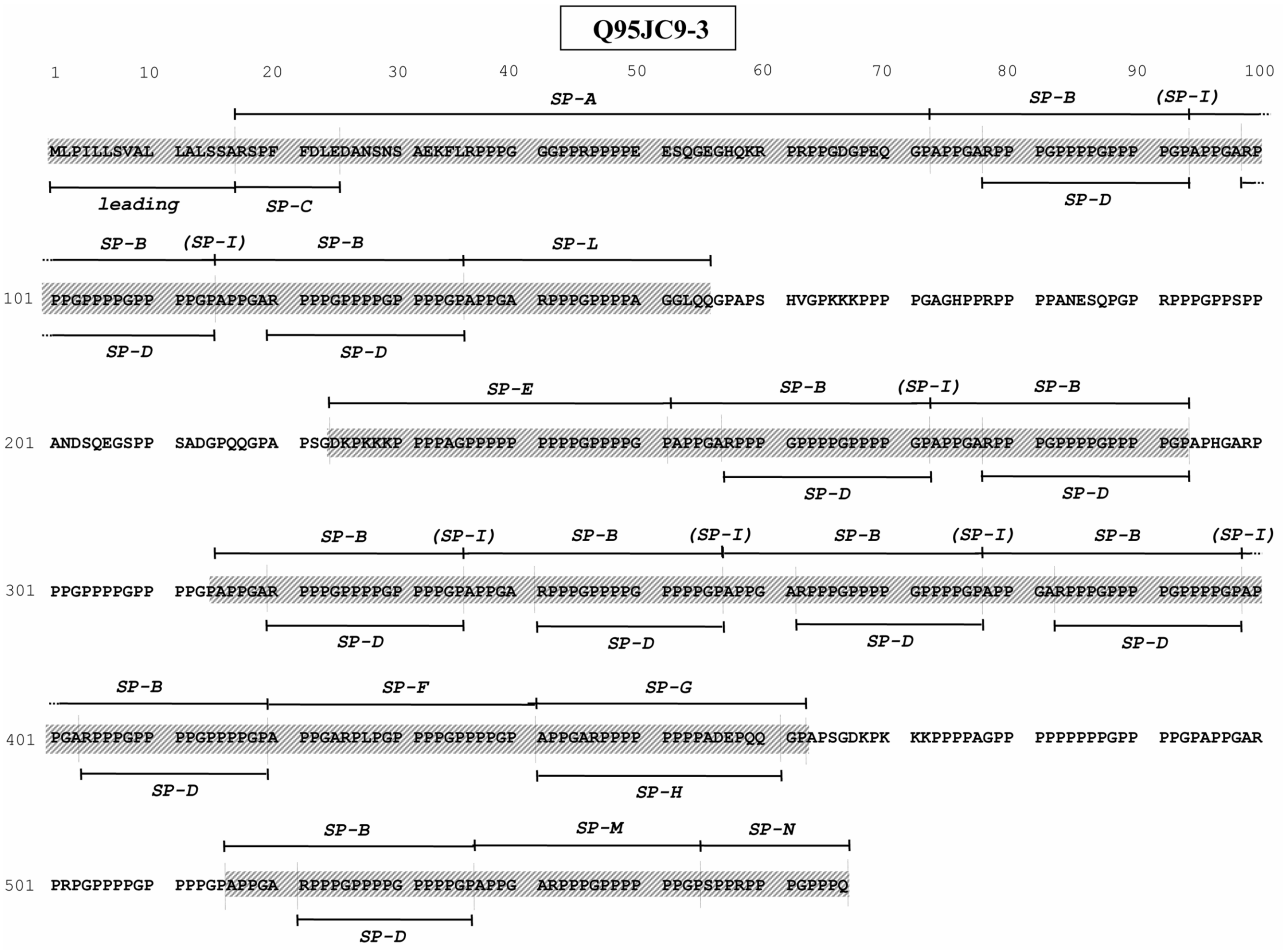


Figure 5. Amino acid sequence of basic proline-rich proprotein isoform **Q95JC9-3**. The regions coding for the identified peptides are represented by bar lines with the indication of the peptide's name.

ture being related to their unstructured character [20, 21].

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