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Structural characterization of a new statherin from pig parotid granules‡

Barbara Manconi,[a] Chiara Fanali,[b] Tiziana Cabras,[a] Rosanna Inzitari,[b] Maria Patamia,[c] Emanuele Scarano,[d] Antonella Fiorita,[d] Alberto Vitali,[c] Massimo Castagnola,[b,c] Irene Messana[a] and Maria Teresa Sanna[a]*

This study describes the identification and structural characterization of *Sus scrofa* statherin. HPLC–electrospray ionization mass spectrometry analysis on pig parotid secretory granule extracts evidenced a peptide with a molecular mass value of 5381.1 ± 0.6 Da and its truncated form, devoid of the *C*-terminal Ala residue, with a molecular mass value of 5310.1 ± 0.6 Da. The complete sequence of pig statherin gene was determined by sequencing the full-length cDNA obtained by rapid amplification of cDNA ends. The gene is 549 base pairs long and contains an open reading frame of 185 nucleotides, encoding a 42-amino acid secretory polypeptide with a signal peptide of 19 residues. This sequence presents some typical features of the four statherins characterized till now, showing the highest degree of amino acid identity with bovine (57%) and human statherin (39%). Pig statherin is mono-phoshorylated on Ser-3, while primate statherins already characterized are di-phosphorylated on Ser-2 and Ser-3. This difference, probably connected to the Asp-4 → Glu substitution, suggests the involvement of the Golgi-casein kinase, which strictly recognizes the SX(E/pS) consensus sequence. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cDNA; ESI-MS; Golgi-casein kinase; phosphorylation; salivary granules

Introduction

Human statherin is a 43-residue peptide, secreted by parotid and submandibular glands, that contains a high number of tyrosine residues and is phosphorylated on Ser-2 and Ser-3 in its mature form. One of the primary functions of this multifunctional molecule is connected to its great affinity for calcium phosphate minerals [1], reflecting the ability to inhibit precipitation and crystal growth of HAP from supersaturated solutions of calcium phosphate [2]. The *N*-terminal region, rich in negatively charged amino acids, is important for the inhibition of crystal growth, while the neutral *C*-terminal region regulates the spontaneous precipitation of phosphate salts [1] and inhibits the growth of anaerobic bacteria in the oral cavity [3–5], contributing to the protection of oral mucosa and hard tissues. Recent studies have correlated cancerous lesion diagnosis in the oral cavity with deficiencies of this peptide in salivary glands [6]. Statherin also serves as an important boundary lubricant [7,8].

Statherin sequence was first determined for the human peptide [2] and later on, for two macaques (*Macaca fascicularis* [9] and *M. arctoides* [10]). The hypothesis that the presence of statherin was restricted only to primate [11] failed when genomic DNA analysis showed the presence of a statherin-like gene in cow and of remnant forms in mouse and rat. Cow transcripts were detected by RT-PCR analysis in RNA from salivary gland tissue, whereas no mouse transcripts have been identified yet [12].

During a proteomic analysis of saliva and parotid secretory granules of pig (*Sus scrofa*), we detected a peptide with a molecular mass value of 5381.1 ± 0.6 Da, that resulted to be statherin. Its partial sequence, obtained by MS/MS analysis, and the full cDNA-derived sequence are reported here together with the phosphorylation status and native derivatives detection.

Materials and Methods

Enriched Granule Preparation

Granules were isolated from parotid glands of *S. scrofa* (Landrace breed), which were obtained from the animal house of the Catholic University in Rome. The animals were treated according to the ethical rules approved by the Ethical Committees of the Catholic University, which are in agreement with those accepted by the European Community (86/609/EEC). Pigs were all females, about 90 days old, with a mean weight of about 30 kg.

* *Correspondence to: Maria Teresa Sanna, Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari, Cittadella Universitaria, I-09042 Monserrato, CA, Italy. E-mail: sanna@unica.it*

a *Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari, Cittadella Universitaria, I-09042 Monserrato, CA, Italy*

b *Istituto di Biochimica e Biochimica Clinica, Facoltà di Medicina, Università Cattolica, Largo F. Vito 1, I-00168 Rome, Italy*

c *Istituto per la Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche (C.N.R.), Sez. Roma, Largo F. Vito 1, I-00168 Rome, Italy*

d *Istituto di Clinica Otorinolaringoiatrica, Facoltà di Medicina, Università Cattolica, Largo F. Vito 1, I-00168 Rome, Italy*

‡ *Sus scrofa statherin nucleotide sequence has been deposited in GenBank under the accession number GU332640.*

Abbreviations used: *EGP, enriched granule preparation; ESI, electrospray ionization; HAP, hydroxyapatite; MS/MS, tandem mass; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TFA, 2,2,2-trifluoroacetic acid; XIC, extracted ion current.*

The isolation of granules followed the protocol described in Ref. 13. Protein extract was obtained according to the procedure described in Ref. 14 and is briefly described here. After washing with 0.9% NaCl aqueous solution to eliminate blood, parotid gland was minced into small pieces by using a scalpel and then homogenized at room temperature in a glass Potter-Elvehjem homogenizer with a Teflon pestle as a 5% (w/v) suspension in homogenizing medium (340 mM sucrose, 0.5 mM EDTA, 10 mM HEPES/NaOH, pH 7.4). 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 *g* for 10 min at 4 °C. The supernatant was centrifuged at 2500 *g* for 15 min at 4 °C, and the pellet, corresponding to the crude fraction of secretory granules, was solubilized in 700 µl of 0.2% TFA. The granule fraction was centrifuged at 8000 *g* for 10 min, and chloroform was added to the supernatant (1 : 1, v/v) to remove lipid components. The aqueous phase, representing an EGP, was directly used for further analyses or stored at −80 °C.

Protein Isolation and Identification

EGPs were analyzed by a Thermo Fischer Scientific (San Jose, CA, USA) Surveyor HPLC connected by a T splitter to a PDA diode array detector and to a LCQ Deca XP Plus mass spectrometer equipped with an ESI source and an ion-trap analyzer. The chromatographic column was a Vydac (Hesperia, CA, USA) C8 column (2.1 mm × 150 mm, 5 µm particle diameter). The following solutions were utilized: (eluent A) 0.056% aqueous TFA and (eluent B) 0.050% TFA in acetonitrile–water 80/20 (v/v). The applied gradient was linear from 0 to 55% of B 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 toward the diode array detector and a flow-rate of about 0.10 ml/min toward the ESI source. The diode array detector was set in the wavelength range of 214–276 nm. Mass spectra were collected every 3 ms in the positive ion mode with a capillary voltage of 4.50 kV and capillary temperature of 220 °C. Deconvolution of average ESI mass spectra was automatically performed either using the software provided with the Deca XP instrument (Bioworks Browser) or by MagTran 1.0 software [15].

Tryptic digestion was carried out according to the instructions of the supplier (Pierce Biotechnology, Rockford, IL, USA). The mixture of the freeze-dried HPLC-purified peptides was dissolved in 80 µl of the digestion buffer (0.1 M ammonium bicarbonate, pH 8.0), and after the addition of 40 µl of immobilized trypsin, it was incubated at 37 °C for 8 h. At the end of the digestion, immobilized trypsin was separated by centrifugation at 3000 *g*, and the peptide mixture was frozen to be subsequently submitted to MS/MS analysis. 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.

Mass values determined on digested samples were compared with the expected average and monoisotopic mass values calculated using the PeptideMass program (http://www.expasy.org/tools/peptide-mass.html). Theoretical MS/MS spectra were generated utilizing the MS-Product program available at the Protein Prospector site (http://prospector.ucsf.edu/).

Purified statherin was submitted to automated Edman sequencing using a Procise 610A Protein Sequencer (Applied Biosystems, Foster City, CA, USA).

RNA Extraction and RACE

Total RNA was isolated from 0.2 g of liquid nitrogen-frozen pig parotid gland by TriReagent (Sigma–Aldrich, St Louis, MO, USA) according to the manufacturer's instruction. The quality of purified RNA was verified by the absorbance spectrum from 220 to 300 nm. RT-PCR was carried out using the Enhanced Avian HS RT-PCR Kit (Sigma–Aldrich) with the supplied anchored oligo-$(dT)_{23}$ primer.

Both 3′- and 5′-RACE reactions (5′/3′ Race kit 2nd generation, Roche Diagnostics, Mannheim, Germany) were performed according to manufacturer's instructions, using 1 µg of total RNA. In the 3′-RACE, first strand cDNA was amplified with a set of degenerate primers designed on the basis of a selected piece of the *N*-terminal sequence of pig statherin and optimized by using the most highly expressed codon usage for *S. scrofa*. A 410-bp product was obtained using the recombinant Taq DNA Polymerase (Fermentas, Ontario, Canada), the primer 5′-CAGAAYMGNGAGMGNGAGC-3′, a 19-mer corresponding to the sense strand with 192 redundancies, obtained from the sequence QNRERE, and the anchored oligo-$(dT)_{23}$ universal reverse primer. Touchdown PCR [16,17] was performed using an initial preheating step of 5 min at 94 °C, an annealing step at 65 °C for 45 s, with a decrease of 0.8 °C per cycle for the first 25 cycles, and 30 s at 72 °C. The following ten cycles consisted of 30 s at 94 °C, 45 s at 42 °C, and 30 s at 72 °C. A final extension step at 72 °C for 5 min was included. The 410-bp product obtained with this approach was cloned into the pCRII vector using the TA Dual Promoter Cloning kit (Invitrogen, San Diego, CA, USA) and used to transform competent *Escherichia coli* INVαF′ cells. Five clones were isolated and amplified by direct colony-PCR, using the same PCR procedure reported for the 3′-RACE reactions. An aliquot of the amplification product was electrophoretically analyzed on a 2% agarose gel and plasmid DNAs from positive clones, purified by using Genelute Plasmid Miniprep kit (Sigma–Aldrich), were submitted to direct sequencing. Two sets of antisense gene-specific primers were constructed from the sequence obtained. For the 5′-RACE, RT-PCR reaction was performed using the antisense gene-specific primer 5′-CAGAGAATATCATTACTATTGTTTTA-3′. The first strand cDNA was purified from unincorporated primers and nucleotides by using the High Pure PCR purification kit (Roche Diagnostics). A homopolymeric tail was added to the 5′-end of RT-PCR products, and the obtained cDNA was amplified by PCR using the nested antisense gene-specific primer 5′-GGAAGAATAGGAAGAATCGT-3′ and the anchored oligo-$(dT)_{23}$ primer provided in the RACE kit, according to the protocol supplied. The PCR reaction was carried out with a denaturation step at 94 °C for 5 min, followed by 30 cycles of 15 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C. A final extension step at 72 °C for 10 min was included. The PCR products from the 5′-RACE were cloned into the pCRII vector as described above and the sequence obtained provided the entire open reading frame statherin gene. The wide overlapping of the sequences obtained by 5′- and 3′-RACE allowed defining the complete gene sequence (549 bp).

Dephosphorylation

EGPs were dissolved in 100 µl of 0.2 M Tris/HCl (pH 8.6) and 40 µl of calf intestinal alkaline phosphatase (1 EU/µl) (Boehringer-Mannhein Biochemicals, Indianapolis, IN, USA) were added. Incubation was carried out at 37 °C and after 40 min, the solution was centrifuged at 8000 *g* for 5 min, and immediately analyzed by HPLC–ESI-MS.

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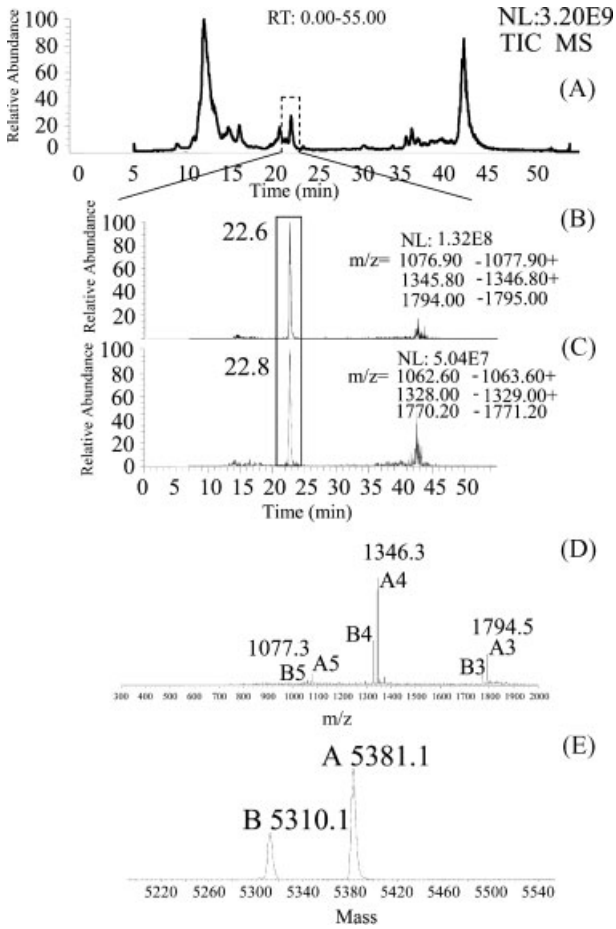

Figure 1. HPLC–ESI-MS profile of EGPs from pig parotid gland. (A) Total ion current profile of EGPs; (B) XIC peak of the 5381.1 Da peptide; (C) XIC peak of the 5310.1 Da peptide; (D) averaged ESI-MS spectra of the two peptides (in the elution range 22.5–23.0 min); (E) deconvoluted ESI-MS spectra showing the two experimental average mass values. RT, retention time; NL, normalization level.

Results

HPLC–ESI-MS analysis of EGPs from pig parotid gland evidenced a peak eluting in the range 22.5–23.0 min that showed the presence of two peptides with *M* average values equal to 5381.1 ± 0.6 and 5310.1 ± 0.6 Da (Figure 1). HPLC–ESI-MS analysis of EGPs submitted to alkaline phosphatase treatment showed a decrease of 80 Da with respect to the mass values of unreacted peptides, suggesting the presence of a single phosphorylation site in both peptides.

Direct sequencing of the peak eluting at 22.5–23.0 min, performed by automated Edman degradation, allowed determining a single 19-residue *N*-terminal sequence corresponding to DSXDEKHHRKXQNREREHY.

In order to obtain the complete primary structure of the peptides, a set of degenerate primers was constructed on the basis of a selected piece of the *N*-terminal sequence and used to determine the full-length cDNA of the corresponding genes. The analysis of positive clones obtained after 3′ RACE amplification of pig cDNA and cloning of the product with the expected length (approx. 410 bp) provided five identical sequences. The 3′ untraslated region (3′ UTR) was used to design a set of reverse gene-specific primers. By 5′ RACE amplification, the entire 5′ UTR and the open reading frame were determined. The 3′ UTR overlapping of the sequences obtained by 5′ RACE allowed defining the complete gene sequence. The full-length cDNA was 549 bp long and contained an open reading frame of 185 nucleotides that started at the first Met codon ATG and ended with a stop codon TAA. The 5′ and the 3′ UTRs of the gene were 51 and 311 bp in length, respectively. The 3′ terminal of the sequence contained a hypothetical polyadenylation signal AATAAA, 13 bp upstream from the poly(A) tail. The gene sequence was used to predict the amino acid sequence (Figure 2). The coding region encoded a 61-residue polypeptide. The *N*-terminal sequence DSXDE determined by Edman degradation allowed recognizing the 42-amino acid secretory portion starting with the DSSDE sequence and the signal peptide of 19 residues that resulted mainly of hydrophobic residues, similarly to the other signal peptides reported earlier [18] (Figure 2). The analysis of the obtained amino acid sequence showed some typical features (i.e. a high content of tyrosine and proline residues and the *N*-terminus DSS(D/E)E motif) of the four statherins characterized till now, suggesting that the peptide under study was indeed pig statherin. Moreover, the theoretical molecular mass value of 5297.5 Da, calculated from the deduced amino acid sequence, resulted in perfect agreement with the experimental mass value obtained by HPLC–ESI-MS analysis on the dephosphorylated peptide with higher molecular mass value.

cDNA sequencing of the five positive clones obtained by 3′ RACE showed no differences attributable to the presence of allelic forms of the gene. Thus, in order to establish the identity of the 5310 Da peptide, the peak eluting between 22.5 and 23.0 min was subjected to trypsin digestion. Table 1 reports the mass values of the theoretical tryptic peptides obtained by *in silico* digestion of the cDNA-deduced amino acid sequence and those detected by HPLC–ESI-MS analysis. Of the six tryptic peptides expected, four (T3, T4 + T5, T6) showed molecular mass values that corresponded to the theoretical ones, and two peptides (T1 and T2) were not detected, the last one perhaps for its small size. An unexpected peptide, coeluting with the theoretical T6, presented a mass value of 2497.1 Da, 71 Da less than T6 (2568.0 Da). In order to clarify this mass difference, the primary structure of the two peptides was determined by MS/MS analysis. The analysis of the fragmentation pattern of these peptides (Figure 3) highlighted the only difference as the loss of the *C*-terminal Ala in the 2497.1 Da peptide, indicated as T6* in Table 1.

A detailed analysis of the HPLC–ESI-MS profile by XIC searches permitted the identification of minor amounts of the nonphosphorylated form of the entire peptide, with *M* average mass value of 5300.7 ± 0.5 Da. This nonphosphorylated form coeluted slightly before the peak pertaining to the phosphorylated peptide, in perfect agreement with the chromatographic elution of the product obtained by alkaline phosphatase treatment on the phosphorylated peptide (data not shown).

Discussion

Sequence alignment of pig statherin with the four statherins sequences already determined highlighted (Figure 4) that pig statherin has a conserved negative *N*-terminal region and a conserved positive charged central region. The *C*-terminal region displayed three conserved Tyr and three conserved Pro residues, but differed in the absence of four Gln residues that are conserved in the three primate statherins characterized till now. Three of these Gln residues were absent in bovine statherin, which showed


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   1 cctctctcagggcttcagaatttcagcttctctgactggactctgccaaatatgaagctc
                                                   M  K  L      3
  61 tttatctttgcctttattatggctctcatgtttgccatgattaaagcagattcatctgac
       F  I  F  A  F  I  M  A  L  M  F  A  M  I  K  A  D  S  S  D   23
 121 gagaaacatcaccggaaatggcaaaatcgggagagagagcattatggtcgaaatcggcca
       E  K  H  H  R  K  W  Q  N  R  E  R  E  H  Y  G  R  N  R  P   43
 181 tattacccgtatgcaccaaattatcctgcatatccattaaattacccatatgcttaaaac
       Y  Y  P  Y  A  P  N  Y  P  A  Y  P  L  N  Y  P  Y  A  -      51
 241 gctgctcagcaaccacaggacatgattacagaggtttggtgaaaaatcccatttgctttg
 301 tactcacgacgattcttcctattcttcccgctatgtccacttcccagctttcacaacaga
 361 aaagaaaaattcaatgaagtagttacatgaacaccaccagctgaaatcaaatatcagtat
 421 ttttaaaacaatagtaatgatattctctgaactatttgattggtttctactttcctttcg
 481 ctgttaaaatattttgaaaatttaaaaaaaaggtcccgcgcataataaattgctctctca
 541 gcaaaaaaa
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Figure 2. cDNA sequence encoding pig statherin and its derived amino acid sequence. Single-letter amino acid designations of the statherin residues are listed below the corresponding codons. Amino acid residues of signal peptide are underlined. Hypothetical polyadenilation site is in italics.

Table 1. Mass values of *Sus scrofa* statherin tryptic peptides determined by HPLC–ESI-MS/MS

Theoretical tryptic peptides[a]					Experimental tryptic peptides		
			$[M+H]^+$	M	$[M+H]^+$	M	R. time
Fragment	Position	Sequence	Monoisot.	Average	Monoisot.	Average	min
T1	1–6	DSSDEK	680.3		n.d.		
T1(PHOS)	1–6	DSpSDEK	760.2		n.d.		
T2	7–9	HHR	449.2		n.d.		
T3	10–14	KWQNR	731.4		731.2 ± 0.3		8.2
T4	15–16	ER	304.2		n.d.		
T5	17–21	EHYGR	661.3		n.d.		
T4 + T5	15–21	EREHYGR	946.4		946.8 ± 0.4		8.2
T6	22–42	NRPYYPYAPNYPAYPLNYPYA		2567.8		2568.0 ± 0.5	24.6
T6*	22–41	NRPYYPYAPNYPAYPLNYPY		2496.8		2497.2 ± 0.5	24.6

[a] Obtained on the basis of the cDNA-deduced amino acid sequence.

a higher degree of similarity to the porcine (66%) than to the primate peptides (34–46%).

A high-coverage draft release (98% complete) of *S. scrofa* genome has been released in November 2009 by the Swine Genome Sequencing Consortium and the Wellcome Trust Sanger Institute with accession no. CM000812–CM000830. Human and bovine statherin genes are localized on chromosome 4 and 6, respectively. Conversely, we found that the cDNA sequence encoding for pig statherin aligned perfectly (except for 20 nucleotides) on *S. scrofa* chromosome 8, in correspondence to five subsequent regions, separated by wide gaps, that are probably introns. Interestingly, also human statherin gene shows a very high number of wide introns.

The presence of acidic segments is a common motif in proteins controlling biomineralization processes [19]. Because the *N*-terminal negative tail of human statherin is responsible for HAP binding [1], it is conceivable that this function should be conserved in pig statherin.

The role of the basic segment of human statherin in HAP recognition has been recently investigated by studying several mutants of the peptide with single or multiple mutations substituting the basic side chains (in the region 6–13) to Ala [20]. It has been shown that the single mutants showed the maximal binding coverage to HAP at saturation and only a small decrease in binding affinity to HAP crystals, while the mutant with the four basic residues substituted displayed conserved structural and dynamic properties on HAP, but had a substantially reduced binding affinity (5-fold reduction) and a lowered maximal surface coverage. These results suggested a role of the basic segment in reducing repulsive protein–protein interactions, due to the decreased overall (negative) charge of the peptide, thus promoting an overall higher surface affinity and coverage on HAP. The isoelectric point of pig statherin, calculated on the basis of its

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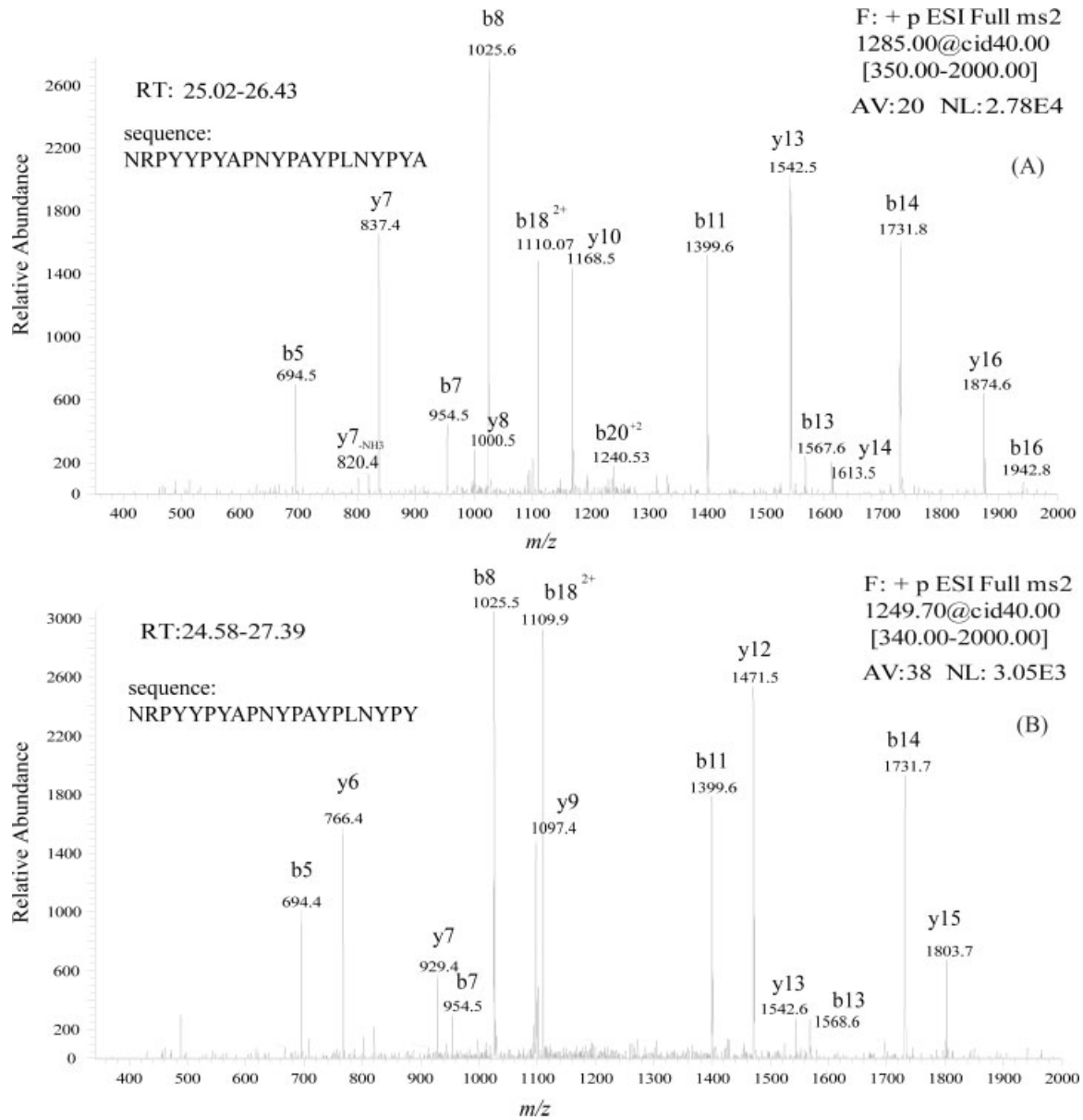

Figure 3. MS/MS spectra performed on the doubly charged ions of pig statherin T6 tryptic peptides. (A) T6 peptide and (B) T6* peptide.

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STAT_HUMAN  MKFLVFAFILALMVSMIGADSSEEKFLRRIGRFGYG-YGPYQ---PVPEQ-PLYPQPYQPQY Q Q Y T F   62
  STAT_PIG  MKLFIFAFIMALMFAMIKADSSDEKHHRKWQNREREHYGRNRPYYPYAPNYPAYPLNY-PYA - - - - -   61
STAT_MACFA  MXFLXFXLXLLXMXXMXXXDSSEEKFLRRLRRFDEGRYGPYQ---PFAPQ-PLYPQPYQPYQ P Q Y - -   61
STAT_MACAR  --------------------DSSEEKFLRRLRRFDEGRYGPYQ---PFVPP-PLYPQPYQPYQ P Q Y - -   42
STAT_BOVIN  MKIFFFAFIMALMVAMIKADSSEEHRLRFNPR---FYYPNQ-QGGYIPSYPAYPYPY-PYP V Q - - -   59
```

Figure 4. Amino acid sequence alignment of statherins by ClustalW software [27]. Each sequence is indicated by its UniProtKB entry name, and its accession number is specified in parenthesis: human (STAT_HUMAN, P02808), pig (STAT_PIG, GU3326640), *Macaca fascicularis* (STAT_MACFA, P02809), *Macaca arctoides* (STAT_MACAR, P14709) and *Bos taurus* (STAT_BOVIN, Q8HY86). Identical amino acids and conservative replacements are shown in black and gray shading, respectively.

amino acid sequence, resulted equal to 9.0, a much higher value with respect to human statherin (pI equal to 6.2), and considering the phosphorylation equal to 7.4 and 4.4, respectively. On the basis of the cited hypothesis of Goobes *et al*. [20], the interaction of pig statherin with HAP should present an increased maximal surface coverage with respect to human statherin considering that all the substitutions involving charged residues (Lys-10 → Arg, Asn-13 → Arg, Arg-14 → Phe, Arg-16 → Tyr, Arg-21 → Pro, Arg-23 → Gln) are located in the central region of the peptide. Further experiments will be devoted to verify this hypothesis.

Recent studies have identified the *C*-terminal (29–43 and 33–39) regions of human statherin as binding domains for *Porphyromonas gingivalis* and *Fusobacterium nucleatum* fimbriae,

respectively [4,21], and these interactions are known to be implicated in causing periodontitis. Since it has been reported that these organisms adhere preferentially to immobilized statherin on HAP surfaces rather than to the free peptide in solution [21,22], it has been hypothesized that surface adsorption may expose statherin receptor sites to bacterial fimbrillin binding. Moreover, the *C*-terminal sequence of human statherin (especially at the level of the sequences QYQQYTF, YQQYTF, QQYTF, QYTF and YTF) has been shown to inhibit the growth of anaerobic bacteria (*Peptostreptococcus* strains, *Fusobacterium necrogenes* and *F. necrophorum*) that may be involved in periodontum diseases [3]. Considering that the *C*-terminal segment of pig statherin is very different from that of human statherin and, in particular, the absence of the QQYTF sequence, pig statherin may offer a model to study if evolution has selected different structures to exploit similar functions.

HPLC–ESI-MS data on tryptic peptides of EGPs from pig parotid gland evidenced the presence of two isoforms of pig statherin differing in the presence of the *C*-terminal Ala residue. In this respect, it is worthwhile to outline that many isoforms of different human salivary peptides, devoid of the *C*-terminal residue, are detectable in human saliva [14] and that different isoforms of human statherin have been described differing for the loss of the *C*-terminal Phe residue (SV1), or of residues 6–15 (SV2), or of both *C*-terminal Phe residue and residues 6–15 (SV3) [23]. These isoforms probably derived from both post-translational modifications and alternative splicing of human statherin gene [24]. Detection of the *C*-terminal truncated isoform in pig parotid EGPs indicates that, in resemblance with data obtained in human, where the different *C*-terminal isoforms are detected in EGPs of both parotid and submandibular glands [14], the *C*-terminal removal is an event occurring before or during granule storage, both in pig and in human salivary glands.

Our data indicate that pig statherin is mono-phosphorylated, while primate statherins are di-phosphorylated on Ser-2 and Ser-3. Automated Edman sequencing identified the residue at position 2 as Ser but failed in recognizing a standard PTH-amino acid as third residue. Furthermore, HPLC–ESI-MS analysis of the tryptic digest of the phosphorylated peptide (Table 1) excluded all other possible phosphorylation sites, represented by eight Tyr residues present at the *C*-terminus. These results, together with the cDNA-derived sequence, indicate that Ser-3 is the only phosphorylation site present in pig statherin.

The different phosphorylation of pig statherin with respect to the primate statherins already characterized is probably related to the Asp-4 → Glu substitution present in the porcine peptide and strongly suggests the involvement of the pleiotropic Golgi-casein kinase acting in many organs and tissues, and responsible for the phosphorylation of many human salivary peptides [25]. This Golgi-casein kinase recognizes SX(E/pS) as the main consensus sequence, and it is therefore not able to phosphorylate Ser-2 residue. It is interesting to note that, as in human, the same enzyme is involved in the phosphorylation of other salivary pig peptides, such as the SP-A proline-rich peptide, characterized in whole saliva and EGPs of pig [26].

Acknowledgements

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