Vol. 197, No. 1, 1993
November 30, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 248-255

AMELOGENIN POST-TRANSLATIONAL MODIFICATIONS: CARBOXY-TERMINAL PROCESSING AND THE PHOSPHORYLATION OF BOVINE AND PORCINE "TRAP" AND "LRAP" AMELOGENINS

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Received October 13, 1993

SUMMARY: TRAP and LRAP amelogenin components were isolated by size-exclusion and reversed-phase HPLC from developing dental enamel. Porcine developing enamel contains TRAP and LRAP components analogous to those of bovine. Amino acid composition and mass spectrographic analyses established that, in both species, the carboxy-terminal sequences of the LRAP components are two residues longer than previously reported for bovine LRAP, and that both the TRAPs and LRAPs contained a single phosphorylated residue. These amelogenin polypeptides were the principal components of the enamel protein lower molecular weight fraction. The LRAP sequence data for both species suggests that the mechanism of amelogenin carboxy-terminal processing may differ significantly from that previously suggested.

Developing, secretory stage, enamel contains a complex of amelogenin proteins which include components ranging in size from 5-25 kDa (1-3). This protein complex arises through the expression and secretion of a family of amelogenins derived from multiple mRNAs generated by differential splicing from copies of the amelogenin gene located, according to species, on the X or the X and Y chromosomes (4-9). Subsequent to secretion, and concomitant with the biomineralization of the extracellular matrix, these proteins appear to undergo proteolytic processing and eventual translocation from the mineralizing front (10-12). It is believed that this sequence of secretion, proteolysis and translocation is directly linked to the processes controlling the development and organization of the enamel biomineral phase, however, the mechanisms involved remain obscure. Within this complex of proteins, two classes of amelogenin polypeptides of 5-6kDa in size have been described, and based on their amino acid compositions designated as "TRAP" (tyrosine-rich amelogenin polypeptide) or "LRAP" (leucine-rich amelogenin polypeptide) (13). Amino acid sequencing of bovine and human TRAPs established that this polypeptide is identical with the amino-terminal 44-45 residues of the highly conserved amelogenin sequence, and is predicted to arise by a specific proteolytic cleavage from the parent protein (14-15).

While the bovine LRAP molecule has the first 33 amino acid residues in common with TRAP, the 13 residue carboxy-terminal sequence is different. Initially it was suggested that this polypeptide was processed similar to TRAP, but from a different, and as yet unidentified amelogenin (15). Subsequently, it was shown that the distinctive carboxy-terminal sequence of bovine LRAP corresponded to residues 172-184 of the 197 residue bovine amelogenin raising the possibility that

Abbreviations: TRAP: Tyrosine-rich amelogenin polypeptide. LRAP: Leucine-rich amelogenin polypeptide.

0006-291X/93 $4.00

LRAP arose by alternative splicing of amelogenin gene transcripts (Figure 1). Recently, this interpretation was verified by Gibson et al (1991) who identified a bovine mRNA of ~200bp in size coding for a 59-amino acid residue polypeptide which is believed to arise by alternative splicing to an internal recognition site within exon-6 of the bovine amelogenin gene (5,16). It was suggested that the 46-amino acid bovine LRAP previously described (13) was a product of specific post-translational proteolytic processing of this 59-residue precursor (16).

The present communication reports the isolation and characterization of TRAPs and LRAPs derived from bovine and, for the first time from porcine sources. While the previous amino acid sequence data for the TRAP molecules is confirmed, the LRAPs are shown to be significantly larger than originally reported (13), suggesting a mechanism of post-secretory processing alternative to that previously postulated (14). In addition, each of these polypeptides has been found to contain a single phosphorylated residue. These observations have implications for understanding the functional role of amelogenins and the nature and specificities of the enamel proteases.

MATERIALS AND METHODS

Developing enamel proteins. Bovine enamel was isolated by blunt dissection from unerupted molar teeth of 8-9 month in *utero* fetuses obtained within one hour of slaughter. Proteins were isolated from acetic acid extracts of enamel scrapings as previously described (17). Porcine enamel proteins were similarly isolated from unerupted third molars of 6 month old pigs obtained freshly from slaughter. The acidic protein extracts were desalted on columns of Bio-gel P2 (Bio-Rad Laboratories, Richmond, CA) and lyophilized.

HPLC procedures. Size exclusion HPLC was conducted by isocratic elution of a TSK-G3000SW column (0.7 x 60 cm) eluted with 0.1% trifluoracetic acid at 0.5 ml/minute. Quantita-

(a) The two forms of TRAP, derived by a cleavage at residues 45-46, are almost identical between the two species. (The differences from the bovine sequence are shown in bold).
Bovine:
MPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPYPSYGYEPMGGW 45
MPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPYPSYGYEPMG 43
Porcine:
MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPYTSYGYEPMGGW 45
MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPYTSYGYEPMG 43

(b) Bovine amelogenin sequence of 197 residues (9):
MPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPYPSYGYEPMGGWLHHQIIPVVSQQTPQNHALQ
PHHHIPMVPAQQPVVPQQPMMPVPGQHSMTPTQHHQPNLPLPAQQPFQPQSIQPQPHQPLQPHQPLQ
PMQPMQPLQPLQPLQPQPPVHPIQPLPPQPPLPPIFPMQ**PLPPMLPDLPLEAWPATDKTKREEVD**

(c) Bovine LRAP-59 precursor and derived LRAPs:
MPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPPLPPMLPDLPLEAWPATDKTKREEVD 59
MPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPPLPPMLPDLPLEAWP 48
MPLPPHPGHPGYINFSYEVLTPLKWYQSMIRHPPLPPMLPDLP 43

(d) Porcine amelogenin sequence of 173 residues (25):
MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPYTSYGYEPMGGWLHHQIIPVVSQQTPQSHALQ
PHHHIPMVPAQQPGIPQQPMMPLPGQHSMTPTQHHQPNLPLPAQQPFQPQPVQPQPHQPLQ********
**********PQSPMHPIQPLLPQPPLPPMFSMQ**SL***LPDLPLEAWPATDKTKREEVD

(e) Porcine LRAP-56 precursor and derived LRAPs:
MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPSL***LPDLPLEAWPATDKTKREEVD 56
MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPSL***LPDLPLEAWP 45
MPLPPHPGHPGYINFSYEVLTPLKWYQNMIRHPSL***LPDLP 40

Figure 1. Amino acid sequences of bovine and porcine amelogenins (9, 16, 26) and the sequences for the TRAP and LRAP polypeptides reported in this study.
In (b) and (d) the residues contributing to the alternatively spliced LRAP precursors are shown in **bold** type.
* : indicates deletions as compared to bovine amelogenin.

tive estimation of protein proportions was obtained by area integration of chromatograms monitored at 220 nm. Fractions isolated by size-exclusion were further fractionated by reverse-phase HPLC (Vydac, C4-214TP54 column, Separations Group, Hesperia, CA) eluted with a gradient of 60% v/v aqueous acetonitrile in 0.1% v/v trifluoracetic acid at a flow rate of 0.5ml/minute. Eluants were monitored at 220 nm.

Amino acid analysis. Lyophilized samples from the HPLC separations were prepared by vapor-phase HCl hydrolysis at 150 °C for 60 minutes. For phospho-amino acid determination, samples were subjected to partial acid hydrolysis (4N HCl at 110 °C) for 2, 4 or 6 hours (19). The hydrolyzates were analyzed by reversed-phase HPLC as their PTC-derivatives (18).

Mass spectrometry. Mass spectrographic analyses were conducted by M-Scan Inc. (Westchester, PA), employing either electrospray ionization (VG BIO-Q instrument with quadrupole detection), or by Fast Atom Bombardment (VG Analytical ZAB 2-SE spectrometer) systems. Computation of the mass of polypeptides or their fragments was made using MacBioSpec (PE SCIEX, Thornhill, Ontario.).

Tryptic digestion of bovine LRAP. Bovine LRAP (Figure 3(a), R4) was digested with solid-phase trypsin (SIGMA product # T-4019) in 0.05M ammonium bicarbonate, pH 8.5, 37 °C for 12 hours. The digest was separated from the enzyme by centrifugation and the supernatant lyophilized. The dried digest was dissolved in 0.1% (v/v) trifluoracetic acid and fractionated on the C4 reversed phase column employing a linear gradient of 60% aqueous acetonitrile (0 - 80 % in 60 minutes) at a flow rate of 0.5ml/minute (data not shown). Tryptic peptides (T1 and T3) corresponding respectively to the amino- and carboxy-terminal fragments of the LRAP were isolated and subjected to mass spectrographic and amino acid analyses.

RESULTS

Isolation and purification. A typical chromatogram illustrating the separation of the TRAP/LRAP fraction of bovine enamel by size-exclusion HPLC is shown in Figure 2. These polypeptides occurred as a pair of partially resolved trailing peaks (T6/T7) representing, in this case, about 20%

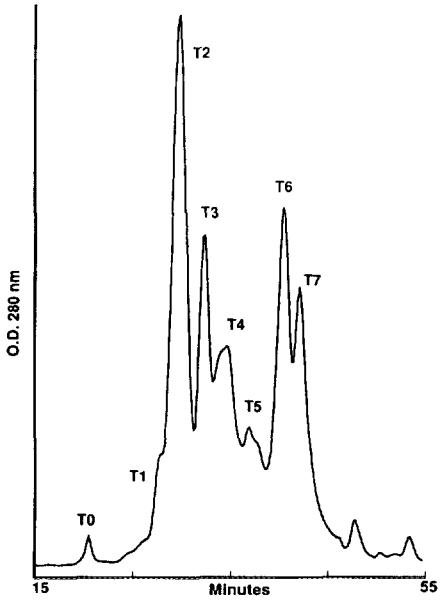

Figure 2. Size-exclusion chromatography of bovine developing enamel proteins. The peaks T6 and T7 were found to contain the lower molecular weight components, TRAP and LRAP. See text for details.

of the total protein applied. Reversed-phase HPLC chromatograms obtained from the separation of the combined T6/T7 (Figure 2) peaks of proteins from both cow and pig are shown in Figure 3. In each case (cow and pig) it was found that four principal chromatographic components (R1-R4 and P1-P4) were present together with a further 4-5 minor constituents. In the case of the bovine sample, chromatographic integration showed that R1-R4 comprised about 86% of the total protein applied. The retention times for the peaks R1,R2 and P1,P2 appeared identical between the species, but the porcine P3 and P4 consistently eluted slightly earlier than their bovine counterparts.

Characterization. Amino acid composition data for the polypeptides R2,R4 and P2,P4 are shown in Table 1. The compositions of R1,P1 and R2,P2 were consistent with that computed for the 45 residue amino-terminal TRAP sequence (Figure 1), while components R3,P3 and R4,P4 were each found to be leucine enriched with compositions consistent with that of an LRAP (13). Mass spectrographic analysis data for these reversed-phase chromatographic components are summarized in Table 2.

The observed mass data for the bovine LRAP components (R3,R4) were significantly larger than was predicted by the previous data (13). Likewise, components P3 and P4 also appeared to be larger than would be predicted for an LRAP derived from the existing porcine amelogenin sequence (25). Computational analysis of these results based on these amelogenin sequences and applying from 1 to 3 phosphorylated residues per molecule demonstrated the following; (i) both the porcine and bovine TRAP components contained a single phosphorylated residue and had amino acid compositions consistent with those previously reported, and (ii) the sizes of the LRAP components could only be reconciled with the observed mass data assuming a single phosphorylated residue, together with an additional Trp-Pro sequence present at the carboxy-terminus (Figure 1).

Mass spectrographic and amino acid analyses (Tables 2 and 3) of the bovine LRAP (R4) tryptic peptide T3, corresponding to the carboxy-terminal fragment, provided a mass value of 1,921 Da; in conformity with the predicted carboxy-terminal sequence; HPPLPPMLPDLPLEAWP, while the amino-terminal tryptic peptide T1 had a overall size of 2,786 Da, consistent with the presence

Table 1
Amino acid compositions of TRAP and LRAP isolates
(Residues/1000)

	TRAP-1				LRAP-1				Bov. LRAP-1, Tryptic peptides			
	Bovine (R2)		Porcine (P2)		Bovine (R4)		Porcine (P4)		N-terminal (T1)		C-Terminal (T3)	
	Found	Sequ.	Found	Sequ.	Found	Sequ.	Found	Sequ.	Found	Sequ.	Found	Sequ.
Asx	30	23	43	47	39	43	70	70	40	42	63	62
Glx	77	68	79	70	65	65	79	70	42	42	65	62
Ser	68	68	47	47	44	43	48	46	33	42	0	0
Gly	113	114	131	116	48	43	59	46	87	83	0	0
His	71	68	79	70	63	65	67	70	85	83	64	62
Arg	25	23	26	23	30	22	22	23	0	0	0	0
Thr	23	23	52	47	22	22	26	23	40	42	0	0
Ala	0	0	5	0	23	22	24	23	0	0	64	62
Pro	226	205	168	186	309	283	243	233	255	250	424	437
Tyr	123	136	142	140	60	65	59	70	81	83	0	0
Val	25	23	23	23	20	22	22	23	42	42	0	0
Met	45	68	54	70	42	65	41	46	44	42	62	62
Ile	40	45	42	47	35	43	38	46	37	42	0	0
Leu	77	68	71	70	150	152	156	163	132	125	259	250
Phe	31	23	17	23	26	22	23	23	44	42	0	0
Lys	24	23	23	23	24	22	23	23	38	42	0	0

Note: Data excludes tryptophan, which was not determined.

Table 2

Mass spectrographic data (Da.) for analyses of bovine and porcine TRAP & LRAP preparations and derived tryptic peptides

Sample*	Found	Predicted#
Bovine LRAP-1 (R4)	5652.5	5653.6
Bovine LRAP-2 (R3)	5055.8	5056.9
Porcine LRAP-1 (P4)	5344.1	5345.2
Porcine LRAP-2 (P3)	4747.3	4748.5
Bovine LRAP-1, tryptic peptide T1	2786.0	2786.2
Bovine LRAP-1, tryptic peptide T3	1921.0	1921.3
Porcine TRAP-1 (P2)	5403.4	5405.1

* Characters in parentheses refer to the reversed-phase HPLC components. T1 and T3 are the amino- and carboxy-terminal tryptic fragments of R4, respectively.
\# Predicted masses assume a single phosphorylated residue in each case, except for peptide T3.

of a single phosphorylated residue within the sequence; MPLPPHPGHPGYINFSYEVLTPLK. Fast atom bombardment mass spectrometry permitted the identification of low levels of fragments from peptide T1, consistent with the presence of a phospho-serine residue at position 16 and partial acid hydrolyzates of R4 showed low levels of phospho-serine.

DISCUSSION

TRAP cleavage and proteolytic processing is specific. Apart from a report on the sequences of human TRAP molecules (21), no other study of these amelogenin polypeptides has been made since the original report (13) which provided the amino acid sequences for bovine TRAP and LRAP[2]. Existing data (13, 26) suggests that the 45-residue TRAP molecule is generated by scission of the parent amelogenin between residues Trp45-Leu[46], and a recent report has described a specific protease in bovine enamel which apparently facilitates this cleavage (27). More enigmatic was the observation that the bovine TRAP-45 was accompanied by a second TRAP molecule (TRAP-43) which was found to be two residues shorter (Figure 1), suggesting that either there existed a second endo-amelogeninase cleaving the parent protein at Gly[43]-Gly[44] or, more likely, that TRAP-43 arose by some specific amino-peptidase action on the TRAP-45 molecule. These earlier observations, taken together with the present data show, for the first time, that in all three species (man, cow and pig), specific post-secretory proteolytic processing of the amelogenins generates two forms of TRAP polypeptides (Figure 1) with carboxy-terminal sequences; -YEPMGGW and -YEPMG which are major components of the lower molecular weight amelogenin fraction (Figure 3).

LRAP sequences suggest an alternative mechanism for amelogenin carboxy-terminal processing. The original 1981 study reported the amino acid sequences of two forms of LRAP in bovine enamel; an LRAP-46 with a carboxyterminal -LPELPLEA sequence which occurred in company with an LRAP-42 (four residues shorter) lacking the carboxy-terminal -PLEA sequence (13). Direct Edman determination of the amino acid sequence of a bovine amelogenin (26) had also shown that the sequence ended at -PLEA. Subsequently, analysis of a bovine amelogenin cDNA

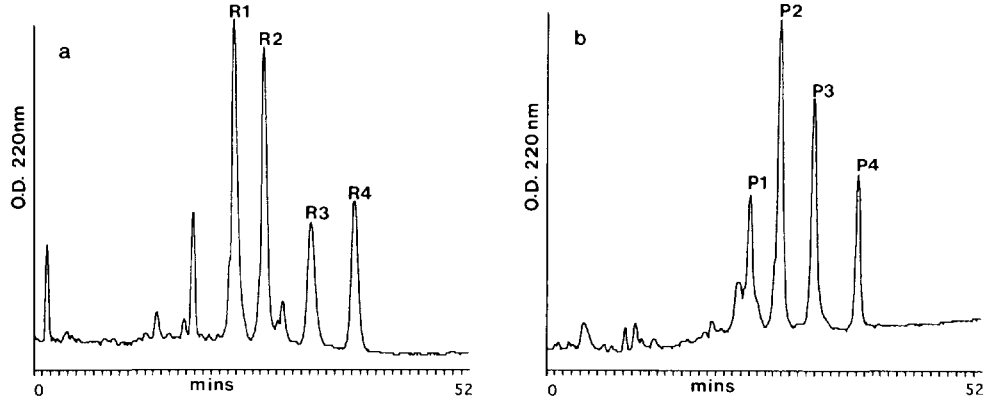

Figure 3. Reversed-phase HPLC profiles for the amelogenin polypeptides isolated by size-exclusion HPLC (Figure 1) from the combined T6-T7 fractions. (a) bovine. (b) porcine. See text for details.

(9) deduced primary structure provided a 197-residue sequence with the hydrophilic carboxy-terminal motif; -PLEAWPATDKTKREEVD; since demonstrated to be almost identical between amelogenins from different species (2). It was suggested that the Ala-Trp bond (bold) in this conserved carboxy-terminal section represented a specific cleavage site for a putative endo-amelogeninase (14). Further, that this cleavage of the hydrophilic "C-teleopeptide" occurs as an early event in the post-secretory processing of the amelogenin which, it is suggested, is directly linked with the biomineralization process (15, 28-29).

The present study shows that the mass-spectrographic data for the bovine LRAP isolates cannot be reconciled with the 46-residue polypeptide previously described (13), but requires the addition of the two additional residues -WP (confirmed by direct sequencing of the tryptic peptide T3). This observation calls into question the significance of the Ala-Trp bond (discussed above) as a specific amelogenin cleavage site, and further suggests that the Edman sequence data originally obtained for the bovine LRAPs were incomplete.

It is now established that the bovine LRAP molecule initially arises by the expression of an alternatively-spliced mRNA coding for a 59-residue polypeptide (Figure 1) which was thought to be processed to a 46-residue LRAP (16). Identification of the two porcine LRAPs, analogous to those found in bovine, provides direct evidence for alternative-splicing of amelogenin gene transcripts in the pig. Further, recent studies of alternative-splicing of murine amelogenin mRNA have also identified a clone containing the coding region for this 59 residue polypeptide (5). Whether amelogenin carboxy-terminal post-secretory processing occurs through a series of specific endo-peptidase cleavages, or whether it is mediated by a progressive carboxy-peptidase action, is presently unclear. However, the absence of multiple other amelogenin polypeptides in the lower molecular weight chromatographic fractions (Figure 3) and the new data for the LRAP sequences appear to favor a carboxy-peptidase mechanism, possibly inhibited by carboxy-terminal proline residues (Fig. 1).

Bovine and porcine amelogenins contain a single phosphorylated residue within the amino-terminal region. The issue of amelogenin phosphorylation has been controversial and difficult of resolution (2). Based on an extended series of studies of bovine enamel proteins, Glimcher and co-workers have reported levels of amelogenin phosphorylation ranging from 1 to 3 residues per mol-

ecule (30-31). We reported non-stoichiometric levels of phosphorylation in bovine TRAP although consistent with a single phosphorylated residue per molecule (13). Takagi and colleagues reported Ser16 of the bovine amelogenin to be phosphorylated, although no experimental detail was provided (26). Subsequently, studies in our laboratory (unpublished data) employing either radio-labeling of in *vitro* organ cultures, or amino acid analyses of partial hydrolyzates failed to confirm this view (2). However, the mass-spectrographic data reported in this communication is clearly consistent with the presence of a single phosphorylated residue in both the TRAP and LRAP isolates. The analyses of the bovine LRAP and its tryptic peptides (T1 and T3) establishes the phosphorylated site as being within the T1 sequence and suggests that this locus is Ser^{16} as previously reported (26). The significance of one phospho-serine to amelogenin function, remains to be explored.

Conclusions. This study demonstrates that LRAP and TRAP are important components of bovine and porcine mineralizing enamel. Although LRAP was not isolated in analyses of human enamel proteins (20), an mRNA coding for the LRAP precursor has recently been identified in the mouse (5). The isolation of the porcine LRAP molecules, as reported in the present study, establishes that alternative-splicing of amelogenin transcripts must also occur in the pig. The new data presented for the sequences of the LRAPs suggests that the proposed specific carboxy-terminal processing of amelogenins by an endo-peptidase at the conserved Ala-Trp locus (14) may be incorrect and an alternative mechanism based on exo-peptidase activity may be considered.

The mass spectrographic studies reported in this communication have confirmed the presence of a single phosphorylated residue in both porcine and bovine TRAP and LRAP molecules as previously reported (13, 26, 30-31). The functional significance of this single phosphorylated site is, however, unclear. Further, it remains an open question as to the functional role in enamel biomineralization of TRAP and LRAP which appear to be the principal components of the maturation-stage mineralizing enamel matrix (20, 32).

Acknowledgments: We are grateful to Drs. Mark Rogers and John Wagner of M-Scan,Inc. for their helpful advice on the interpretation of the mass-spectrographic analyses and to our colleagues Jim Simmer, Ed Lau, and Harold Slavkin for their valued discussions. This work was supported by NIH, NIDR research grant DE-02848.

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