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Human Amelogenins: Sequences of "TRAP" Molecules

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Summary. The extracellular protein matrix of developing enamel includes a major class of proteins, the amelogenins, which are believed to be concerned in regulating enamel biomineralization. Previous studies have shown the amelogenins of the extracellular matrix to be a complex of proline-rich hydrophobic proteins which, it is suggested, arise through posttranslational and postsecretory processing of a primary ameloblast gene product. More recently, it has been shown that the human amelogenin gene is located on both the X and Y chromosomes raising the possibility that polymorphism at the level of the gene may also contribute to the observed complexity of these enamel matrix proteins. To investigate such possible amelogenin polymorphism in developing human dental enamel, individual samples of human enamel proteins were fractionated by size-exclusion and reversed-phase high pressure liquid chromatography (HPLC). Two tyrosine-rich amelogenin polypeptides (TRAPs) of approximately 5 kDa in size were isolated from an individual human dentition and characterized by automated gas-phase sequencing. These polypeptides were found to be of 42 (TRAP-2) and 44 (TRAP-1) amino acid residues in length; TRAP-2 lacked a carboxy-terminal -Gly-Trp sequence as has previously been described for analogous bovine TRAP molecules. However, residue #25 of the human TRAP-2 sequence was refractory to sequencing, apparently differing from the Trp-25 identified in TRAP-1. These findings suggest (1) two forms of TRAP molecules, differing only by cleavage of a carboxy-terminal dipeptide, are a general feature of human and other mammalian enamel proteins, probably being derived by postsecretory cleavage from the primary extracellular amelogenin; and (2) in human developing enamel four forms of TRAPs may arise either from polymorphism at the level of the gene, or by posttranscriptional alternative splicing of amelogenin mRNAs, coupled with specific post-secretory proteolytic processing.

Key words: Enamel — Amelogenin — Sequence — Human.

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Recent studies have shown that mouse and bovine amelogenin genes (AMEL) are represented in genomic DNA as single, nonrepeated, structural gene sequences [1, 2] and that in man, there are two amelogenin gene loci residing each on one of the X and Y sex chromosomes [2]. Although it is unknown if both of these human amelogenin genes are functional, the possibility of genetic polymorphism contributing to the observed complexity of the human extracellular enamel matrix proteins cannot be overlooked. Also, it has been shown that multiple immunoreactive amelogenin polypeptides are present in cell-free translation preparations of ameloblast mRNAs for several mammalian species including hamster, mouse, and cow [3–5]. It is not presently known if there is colinearity or correspondence in the number of mRNAs and their encoded amelogenin polypeptides produced in any single mammalian species or if there is polymorphism among amelogenins within an individual. Direct analysis of mRNA translation products from human ameloblasts has been difficult to obtain and interpret [6] and we have therefore approached the problem of the characterization of the human amelogenin gene products by analyzing proteins isolated from individual fetal human dentitions.

Chromatographic and electrophoretic studies of the matrix of fetal enamel from several mammalian species have shown striking molecular heterogeneity among the amelogenin proteins [7, 8] and it has been suggested that this complexity may arise through a combination of posttranslational modifications (e.g., glycosylation and/or phosphorylation) as well as from the postsecretory proteolytic processing of the precursor amelogenin molecule [5, 9]. Although it is known that there is a high degree of homology of amelogenin sequence between species, including an identity of sequence for a conserved amino-terminal motif of some 33 amino acid residues [10, 11], further complexity might also occur through polymorphism either at the level of the gene, or from alternative splicing of messenger (mRNA) from a single amelogenin gene [12]. Such polymorphism of amelogenin mRNAs has been indicated in both studies of ameloblast mRNA [3, 13] and in amino acid sequencing of amelogenin polypeptides [10].

Within the complex of amelogenin proteins identified in the fetal enamel matrix, the distinctive lower molecular weight (5–6 kDa) "tyrosine-rich amelogenin polypeptides" (TRAPs) have been shown to have amino acid sequences identified with the amino-terminal section of the higher (20–27 kDa molecular weight) amelogenins [10, 11]. This "TRAP sequence" is distinctive in that it contains all six tyrosine residues found within the larger parent amelogenin and is highly conserved between mammalian species [7, 10, 11]. Further, it appears probable that these TRAP molecules originate from specific postsecretory proteolytic cleavages from the larger precursor amelogenin molecule, a process that may be linked to the mechanism of enamel matrix biomineralization [7, 14].

In the case of human developing enamel, only partial amelogenin characterization and amino acid sequence data for the TRAP molecules has been previously reported [11, 15, 16] and no other data for the amino acid sequence of human amelogenin is presently available. In order to further characterize human amelogenin and to investigate the origins of multiple posttranslational and postsecretory amelogenins in developing human enamel, studies were designed to isolate and characterize TRAP molecules from individual human fetal dentitions.

In this paper we report (1) the characterization of human fetal enamel proteins using a combination of size-exclusion and reversed-phase HPLC; (2) the identification of four putative TRAP components in fetal human enamel and the isolation and amino acid sequences for two of these TRAPs obtained from a single fetal human dentition; and (3) results suggesting that differences in primary structure may exist between human TRAP molecules within the same individual. Collectively, these data indicate that the complex of TRAP structures identified in individual human dentitions may arise either through polymorphism at the level of the gene and/or by alternative mRNA splicing, coupled with specific postsecretory proteolytic processing.

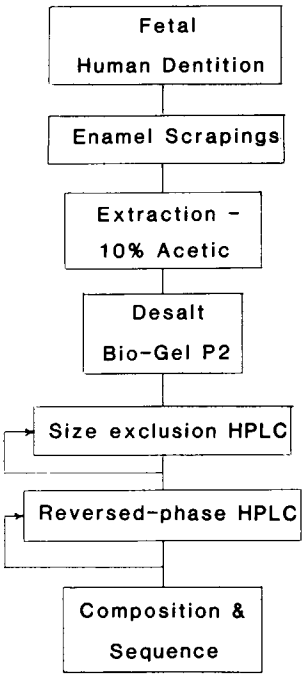

Fig. 1. Scheme for the isolation of human fetal enamel matrix and the purification of TRAP molecules.

Materials and Methods

Isolation of Fetal Human Enamel Proteins

Scrapings of developing human enamel were obtained from unfixed fetal human dentitions of ages ranging from 33 to 36 weeks *in utero*, as previously described [15]. Tissues were generally obtained within 12 hours of fetal demise through University of Southern California, "Human Subjects Review Board," approved protocols. The enamel matrix preparations were demineralized by extraction with acetic acid (10% v/v) at 4°C. Extracts were clarified by brief centrifugation (10,000 rpm, 10 minutes) and desalted by gel exclusion chromatography (Biogel P2 column, 1.5 × 25 cm, eluted with 0.5% (v/v) formic acid). The void volume fraction including all polypeptides of sizes greater than about 1,800 daltons was then lyophylized.

Size Exclusion Chromatography

Samples of demineralized human enamel proteins were fractionated by size exclusion high performance liquid chromatography (HPLC) ('Ultropac' TSK-G3000SW column, 0.7 × 60 cm, Phar-

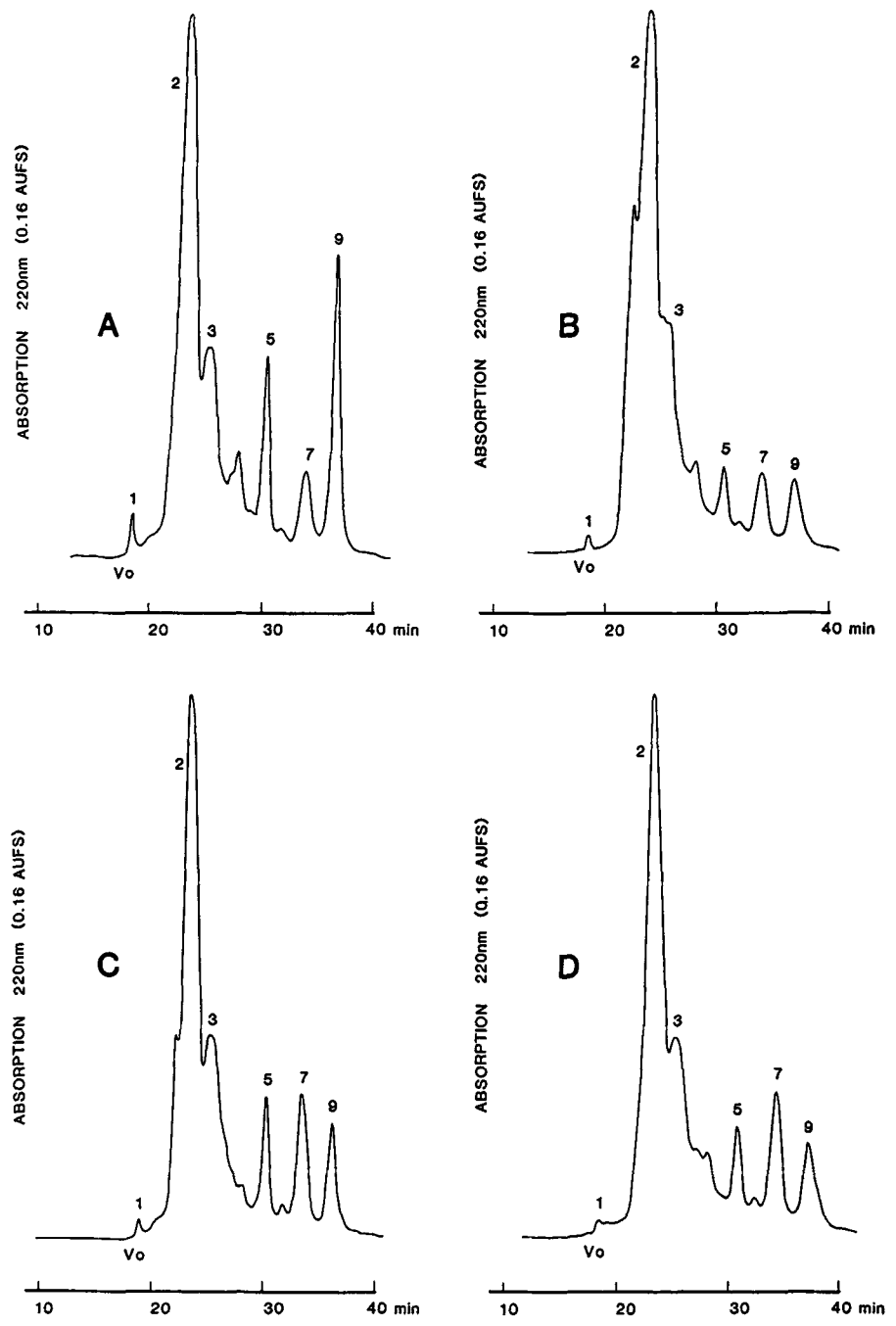

Fig. 2. Comparative size-exclusion HPLC fractionation of human enamel matrix proteins (TSK-G3000SW column eluted with 0.1% (v/v) TFA, 0.5 ml/minute). **(A)** 33 week, male; **(B)** 36 week, male; **(C)** 33 week, female; **(D)** 36 week, female.

macia LKB Biotechnology Inc, Gaithersburg MD) at a flow rate of 0.5 ml/minute in 0.1% (v/v) trifluoracetic acid (TFA). Fractions of interest were further purified by re-chromatography on the TSK-3000 column.

Reversed Phase HPLC

Fractions from the TSK-3000 size exclusion chromatography were further purified by reversed-phase HPLC (VYDAC C4-214TP54, Separations Group, Hesperia, CA; or an Aquapore PH-300, Brownlee Labs, Santa Clara, CA), eluted with gradients of acetonitrile in 0.1% TFA (see figure legends for details).

Amino Acid Analysis

Samples of purified enamel protein were hydrolyzed (6N HCl at 110°, 18–24 hours) in nitrogen-flushed sealed glass ampules. The hydrolysates were analyzed, following pre-column derivatization with phenyl-thioisocyanate, by reversed-phase HPLC [17, 18].

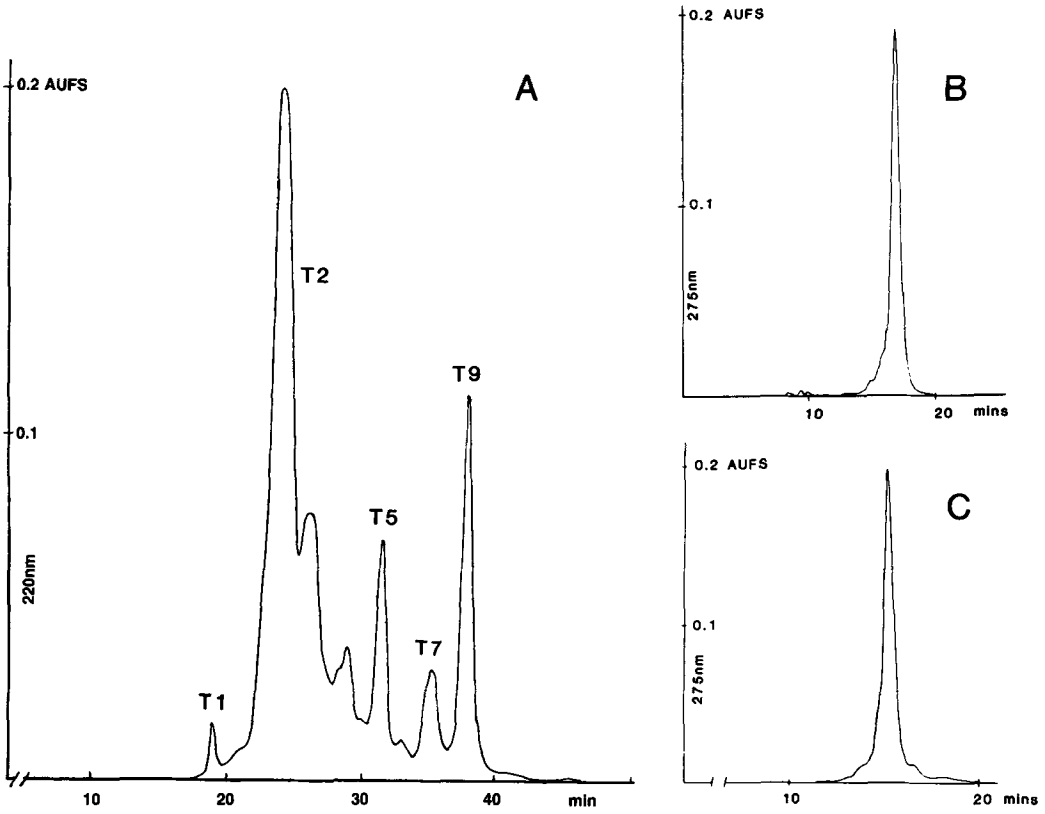

Fig. 3. Isolation of human TRAPs. (A) Size-exclusion HPLC (TSK-G3000SW column, eluted at 0.5 ml/minute with 0.1% (v/v) TFA). Sample: 33 week, male. T2 = main amelogenin complex, T5 = TRAP-2, T9 = TRAP-1. (B and C) Reversed-phase HPLC of TSK3000 peak T9 (TRAP-1) and T5 (TRAP-2). VYDAC-C4 column. Gradient elution; A = 0.1% trifluoracetic acid, B = acetonitrile:water (60:40 v/v), containing 0.1% TFA. Gradient; 60–80% B in 30 minute at 1.2 ml/minute. AUFS = absorption units full scale.

Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoretic analysis of the enamel proteins was carried out using the tris-urea-borate system in 15% acrylamide gels, as previously described [19].

Automated Edman Sequence Determination

The amino acid sequence of HPLC-purified enamel polypeptides was determined using a gas-phase automated sequencer (Applied Biosystems Model 470A) located at the University of Southern California Comprehensive Cancer Center, Microchemical Core Facility, and the phenylthiohydantoin derivatives were analyzed by on-line reversed-phase HPLC.

Results

The isolation and purification procedure used in the preparation of the human amelogenins is summarized in the scheme illustrated in Figure 1. This strategy permitted the rapid isolation of enamel matrix proteins, without contamination from dentin-derived material, and the isolation by size-exclusion and reversed-phase HPLC of two amelogenin polypeptides (TRAP-1 and TRAP-2) which were subjected to automated sequence determinations.

HPLC size-exclusion fractionations (Fig. 2) of representative specimens of acetic acid extracted human enamel proteins, [33 weeks (A,C) and 36 weeks (B,D) *in utero*, male (A,B) and female (C,D)], showed a characteristic and reproducible profile for all samples. A major peak, 20–25 KDa in size and accounting for about 80% of total protein, comprised the principal amelogenin complex (peak 2) and was followed by a group of 4–6 peaks of lower molecular weight components which included the tyrosine-rich amelogenin polypeptides (peaks 5–9) which are the subjects of this study. A small void volume peak (peak 1), presumed to include both enamelins [20] and other higher molecular weight components, was seen in all cases and accounted for 1–2% of sample protein (estimated by 220 nm absorption).

Variation in chromatographic profiles between enamel protein extracts obtained from different in-

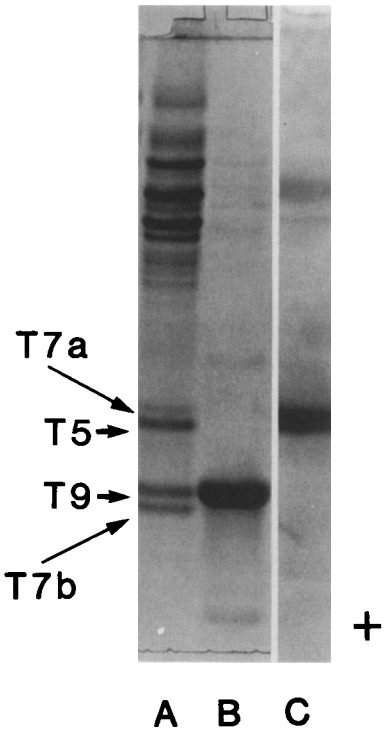

Fig. 4. Tris-borate-urea PAGE of human enamel matrix proteins and of fractions isolated by HPLC. **(A)** Total human enamel proteins identifying the two TRAP molecules sequenced in this study (T5 = TRAP-2, T9 = TRAP-1). Note also (track-A) the presence of the two other putative TRAPs T7a,b (see discussion). **(B)** Size exclusion HPLC fraction "T9" (Fig. 3A), partially purified by reversed-phase HPLC (human TRAP-1). **(C)** Size exclusion HPLC fraction "T5" (Fig. 3A), partially purified by reversed-phase HPLC (human TRAP-2).

Table 1. Amino acid composition of human TRAP-1 (Mean of 3 analyses)

Residue	Res/1000	Res/mol[a]	Sequence
Asp	18	0.71	1
Glu	71	2.87	3
Ser	62	2.46	3
Gly	116	4.68	5
His	49	1.97	2
Arg	24	0.95	1
Thr	35	1.42	1
Ala	0	0.00	0
Pro	248	10.00	10
Tyr	135	5.44	6
Val	27	1.07	1
Met	35	1.42	2
Ile	49	1.98	2
Leu	78	3.16	3
Phe	32	1.29	1
Lys	21	0.83	1
Trp	Not determined		

[a] Normalized to proline = 10 res/molecule

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                    10        20        30        40
(a) MPLPPHPGHPGYINFSYEVLTPLKWYQS - IRPPYSPYGYEPMGGW
(b) MPLPPHPGHPGYINFSYEVLTPLK ? YQS - IRPPYSPYGYEPMG
                                   *
(c) MPLPPHPGHPGYINFSYEVLTPLKWYQS MIRHPYSPYGYEPMGGW
                                   *
(d) MPLPPHPGHPGYINFSYEVLTPLKWYQS MIRHPYSPYGYEPMG
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Fig. 5. Comparison of human (this study) and bovine [11] TRAP sequences. **(a)** Human TRAP-1 (T9). **(b)** Human TRAP-2 (T5) (deletion shown as −). **(c)** Bovine TRAP-1. **(d)** Bovine TRAP-2 (* = difference at residue #32). Note that residue #25 in human TRAP-2 **(b)** was refractory to automated sequence determinations.

dividual human dentitions was seen, only with respect to the relative proportions of the several chromatographic peaks, especially in the case of the lower molecular weight components (Fig. 2, peaks 5–9), no developmentally related pattern being discernable within this 33–36 week time span.

The steps of purification for the isolation of the amelogenin TRAP-1 and TRAP-2 polypeptides are shown in Figure 3. The peaks labeled T5, T7, and T9 in the size-exclusion chromatogram (Fig. 3A) were each characterized by amino acid composition as TRAPs, as previously described [8]. Repeated reversed-phase HPLC of peaks T9 (Fig. 3B) and T5 (Fig. 3C) yielded purified preparations, each comprising a single, fast-migrating band in tris-borate-urea PAGE (Fig. 4). Samples of the purified T5 and T9 components were then subjected to amino acid analysis (Table 1) and automated sequence determinations.

Amino acid analyses of peak T7 (Fig. 3A) suggested that this was also a TRAP component, and subsequent reversed-phase HPLC (not shown) and tris-borate-urea PAGE showed T7 to contain two tyrosine-rich components, identified with the electrophoretic bands T7a and T7b shown in Figure 4. Partial sequence determinations of these two T7 components (T7a, 22 residues and T7b, 15 residues) established the same amino-terminal sequence as found in T5 and T9. However, the separation and isolation of the T7 components proved difficult and they were not further characterized in this study.

Amino acid sequence determinations showed the T9 (TRAP-1) component to comprise 44 amino acid residues (Fig. 5a) with a carboxyterminal-Met-Gly-Gly-Trp motif, identical to the 45 residue sequence previously described for the bovine TRAP-1 (Fig. 5c) [10], with the exception of a deletion of a methionine at residue #29 and a substitution of proline for histidine at residue #32 (see Fig. 5). These data constitute the longest amino acid sequence presently available for human amelogenin and, it is suggested by analogy with other species,

Table 2. Recovery of PTH-amino acids in cycles 23–27 during gas-phase sequencer analyses of human TRAP-1 and TRAP-2

	Picomoles recovered									
	TRAP-1					TRAP-2				
Cycle #	23	24	25	26	27	23	24	25	26	27
Leu	**827**[a]	801	500	301	193	**1097**	896	444	257	184
Lys	6	**676**	647	443	237	n.d.	**731**	618	305	179
Trp	5	6	**313**	292	185	n.d.	32	29	n.d.	n.d.
Tyr	235	267	248	**870**	867	169	236	218	**788**	664
Gln	n.d.	60	85	95	**567**	53	53	100	85	**629**

[a] Residues identified in each cycle are shown in bold

that it represents the amino-terminal segment of the 20–25 kDa size human amelogenin [3, 11, 21, 22].

The T5 (TRAP-2) component had a sequence (Fig. 5b) two residues shorter than T9 (with a carboxyterminal -Met-Gly sequence), hence being analogous to the bovine TRAP-2 molecule described by Fincham et al. [10] (Fig. 5d). However, residue #25 of T5 could not be identified, and there was no evidence for a tryptophan residue at this position, as was found in the T9 preparation.

Phenylthiohydantoin (PTH) amino acid recoveries (total picomoles found) for sequencer cycles #23–#27 are shown in Table 2. It can be seen that whereas tryptophan was unequivocally identified as residue #25 for human TRAP-1 (T9) with a substantial "carry-over" of the hydrophobic residue being seen in subsequent cycles, no such residue was found for the TRAP-2 (T5) sample and a positive identification of residue #25 could not be made, although the remainder of the 42 residue sequence was readily obtained. Amino acid analyses for T5 samples (not shown), confirmed the lower glycine content (4 residues/molecule as compared with 5/molecule for T9), but were otherwise equivocal. Two subsequent analyses of the T5 (TRAP-2) component from the enamel matrix of other individuals also failed to reveal the identity of residue #25 in the automated gas-phase sequencer.

Discussion

Fincham et al. [11] also identified four putative TRAP molecules and reported partial sequence data for two human TRAP preparations isolated by gel exclusion chromatography (Bio-Gel P30) of acetic acid extracts of human fetal enamel scrapings. Their analyses (sequenced to 33 and 30 residues) identified tryptophan at residue #25 of both human TRAP samples and are in agreement with the TRAP-1 (T9) sequence reported here, apart from the tentative identification of methionine as residue #30. Methionine was not identified at this position in the present study.

The cause of the failure of the determination of residue #25 in the human TRAP-2 (T5) sequence is unclear. However, automated Edman sequencing provided no evidence for tryptophan at this position, despite a clear determination being made for Trp-25 of TRAP-1 (Table 2). The negative result obtained with TRAP-2 may arise from the presence of an unknown posttranslationally derivatized (glycosylated or phosphorylated) residue at this position. However, no such modifications of tryptophanyl residues in proteins are known to the writers.

The size exclusion HPLC profiles (Figs. 2 and 3) reveal a chromatographic anomaly in which the shorter (42 residue) polypeptide TRAP-2 (T5) is eluted before the longer (44 residue) TRAP-1 (T9) molecule. Similar behavior has been previously noted for bovine TRAP preparations chromatographed on "soft-gel" columns (e.g., Biogel P30 [8]), and is presumed to arise from hydrophobic interactions with the column packing, perhaps accentuated, in this case by the carboxy-terminal tryptophan residue of the TRAP-1 sequence. (Subsequent experience with size-exclusion HPLC of enamel proteins on silica-based columns eluted with TFA have shown variability in column performance probably attributable to such hydrophobic interactions.)

Previous sequence determinations made on the bovine lower molecular weight (Mr. 5000 kDa) enamel proteins also revealed the presence of two forms of the TRAP molecule, differing only in that the 43-residue bovine TRAP-2 lacked the carboxy-terminal -Gly-Trp dipeptide present in the TRAP-1 structure [10]. The observation reported in this study of a similar situation in the human enamel proteins suggests a common mechanism for the origin of these TRAP structures. The failure to identify a Trp-25 residue in the human TRAP-2 sequence raises the possibility that the TRAP-2 se-

quence could arise by cleavage from a parent amelogenin protein distinct from the TRAP-1 precursor suggesting either (1) separate mRNAs resulting from alternative splice junction selection from a single amelogenin gene [20], or (2) separate transcription from one of the two amelogenin gene loci, recently identified on each of the human X and Y-chromosomes [2].

The observation (Fig. 4) that preparations of human enamel matrix contain four TRAP structures (identified with chromatographic peaks, T5, T7, and T9; Fig. 3), together with our finding that T5 (TRAP-1) and T9 (TRAP-2) apparently differ in their primary structures at residue #25, suggests that the origin of these lower molecular weight amelogenin polypeptides may be more complex than hitherto suggested [7, 10].

We suggest that, in the human, two forms of amelogenin yield two forms of TRAP-1 polypeptides which are then subject to postsecretory proteolytic cleavage of the carboxyterminal Gly-Gly bonds each yielding two TRAP-2 structures. Such complexity could arise either from alternative splicing from a single gene or from polymorphism of the human amelogenin gene [25]. However, present data do not permit us to distinguish between these two possibilities and confirmation of this hypothesis will require further characterization of the complex of human TRAP polypeptides.

The view that polymorphism among amelogenins may occur is supported by previous sequence data [10] which identified bovine leucine-rich amelogenin polypeptides (LRAPs) which were subsequently found not to be colinear with the amino acid sequence of the 27 kDa bovine amelogenin as determined by amino acid sequencing [22] or by interpretation of a cDNA sequence [23]. Further, Young et al. [13], in studies of heterogeneity in bovine mRNA, reported the presence of a 300 bp transcript which was insufficient in length to encode the 27 kDa amelogenin. These authors suggested that this mRNA class might encode a smaller amelogenin protein, such as the LRAP sequence.

Amino acid sequence data for murine, porcine, and bovine amelogenins deduced, either from cDNA data or from chemical sequencing, have revealed a high degree of inter-species homology [22, 23]. However, cell-free translation products of ameloblast mRNA preparations, isolated from several species, have all yielded multiple translation products when immunoprecipitated by amelogenin specific antibodies [5, 6, 24]. The structural relationships between these amelogenins are presently unknown, but further characterization of the TRAP (and possibly LRAP) components of the human enamel matrix will assist in the study of amelogenin polymorphism and its possible implication in genetically related abnormal enamel biomineralization.

In conclusion, our analyses of enamel proteins from both fetal human males and females have not yet been extended to identify possible sex-linked differences. Such studies would be of significance in view of the recent localization of amelogenin gene loci on both X and Y human chromosomes [2]. Analyses of the human amelogenin polypeptides have identified 44- and 42-residue TRAP structures as the (presumed) amino terminal sequences of the 20–25 kDa amelogenin from the human extracellular enamel matrix. Comparison of the human TRAPs with the analogous bovine sequences (or with the amino terminus of the mouse [21] or pig [26] amelogenin sequences) identifies these molecules as a common feature of the mammalian enamel matrix. Finally, the refractory behavior to sequencing of residue #25 in the human T5 (TRAP-2) preparations, suggesting a difference in primary structure, uncovers a potential polymorphism of human amelogenins within a single individual which may arise either at the level of the gene and/or through alternative splicing, as has also been implicated in studies of the bovine amelogenins. The possible significance of this polymorphism of amelogenins in developing human enamel to genetically related abnormalities of enamel are presently obscure, but noteworthy in view of the presumptive role of the amelogenins in enamel formation and biomineralization.

Note Added In Proof

We have recently obtained partial amino-terminal sequence data for a 20 kDa human amelogenin from a different human specimen, which confirmed the TRAP-2 sequence as shown in Figure 5(b), including the deletion of a methionine residue at position #30. Again we were unable to identify the amino acid residue at position #25.

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