

IN VITRO STUDIES OF THE PROTEINASES WHICH DEGRADE AMELOGENINS IN DEVELOPING RAT INCISOR ENAMEL

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**INTRODUCTION**

Much attention has focused recently on proteinases which appear to hydrolyze amelogenins extracellularly during enamel development<sup>1-4</sup>. Current evidence suggests that (a) more than one type of proteinase, including at least one serine proteinase and two metalloproteinases, may be present in developing enamel<sup>1-4</sup>, and (b) there is a change in the proteolytic profile over the course of amelogenesis such that an endoproteinase not evident in secretory stage enamel becomes active in maturation stage enamel<sup>1,2,4</sup>. While there is agreement that one, or possibly two, serine proteinase(s) with trypsin-like properties may degrade amelogenins<sup>1,3,4</sup>, there has been considerable disagreement concerning the molecular weight, pH optimum, and cation requirement of the enzyme(s)<sup>1-4</sup>. The purpose of this study was to learn more about the basic properties of enamel proteinases using enzymograms<sup>1,2</sup> and an *in vitro* assay that allowed the amelogenin degrading activity of the enzymes to be visualized independently of the cells (enamel organ) which normally cover the enamel<sup>4</sup>. We were particularly interested in determining what would happen if enamel enzyme extracts from one stage of amelogenesis were incubated with amelogenins taken from a different stage of amelogenesis.

**MATERIALS AND METHODS**

Thirty-nine male Wistar rats weighing 100 g were anesthetized with ether and decapitated. The hemimandibles were removed, and the enamel organ covering each incisor was exposed and wiped from the tooth with gauze pads moistened with ice-cold saline (0.9%). Individual 2- to 3-mm-long pieces of developing enamel were dissected from the secretory (S) and early (M<sub>E</sub>) and midmaturation (M<sub>M</sub>) stages of amelogenesis on each incisor (Fig. 35:1) and processed for *in vitro* incubation and for electrophoresis of extracted enamel proteins (Fig. 35:2) as described previously<sup>4</sup>. In some experiments, enamel samples were boiled or exposed to PMSF (0.1-2 mM) prior to incubation at 37°C and pH 7.5 in sterile microfuge tubes, or they were incubated without pretreatment at 37°C and pH 7.5 in extraction buffer prepared with either benzamidine, TLCK, TPCK, α-macroglobulin, bestatin, elastatinal, E-64, phosphoamidon, pepstatin A, aprotinin, leupeptin, trypsin-chymotrypsin inhibitor, or SDS and β-mercaptoethanol (Figs. 35:4-6). In other experiments enamel samples were incubated at 20°C in normal extraction buffer, or at 37°C in extraction buffer prepared with sodium acetate (pH 5.0) or Tris-HCl (pH 9.0)(Figs. 35:4-6). As well, some of the S and M<sub>E</sub> enamel samples were initially boiled, then incubated at 37°C and pH 7.5 with unboiled M<sub>M</sub> enamel extracts (Figs. 35:8-9). Lastly, fresh enamel samples, and freeze-dried enamel organ samples<sup>4</sup>, from the secretory (S) and maturation (M<sub>E</sub> + M<sub>M</sub>) stages were homogenized in extraction buffer<sup>4</sup>. The homogenates were divided into two equal portions one of which was treated for 1 hour with cold 5% TCA and centrifuged<sup>2</sup>. The precip-

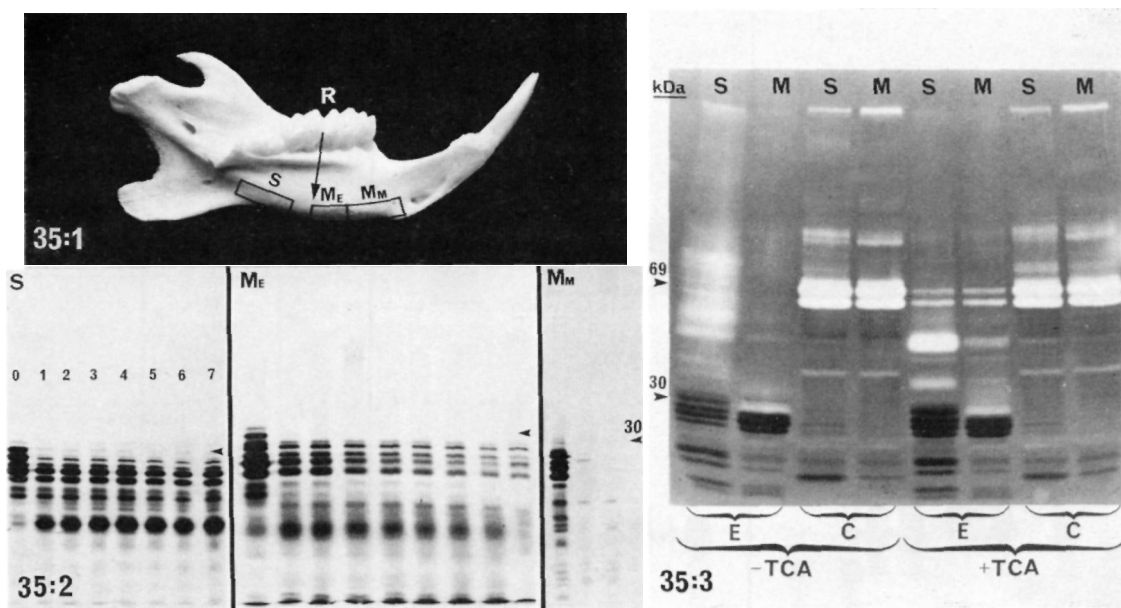
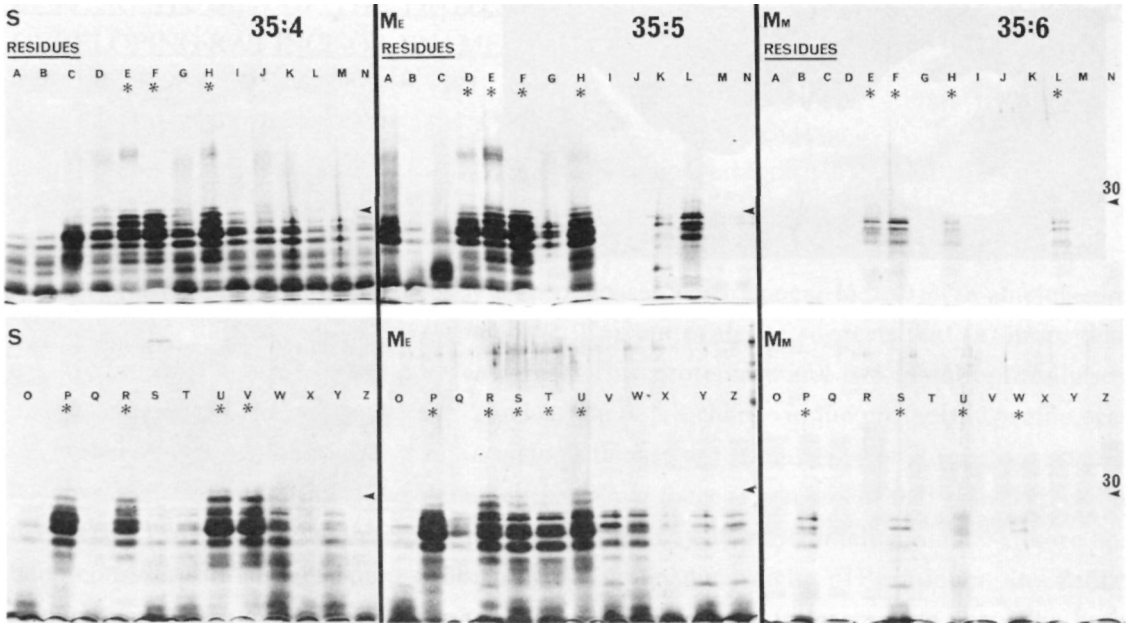


Fig. 35:1. Mesial view of left hemimandible showing locations where pieces of secretory (S), early maturation ( $M_E$ ) and midmaturation ( $M_M$ ) stage enamel were removed from rat incisors relative to a molar reference line (R).  
 Fig. 35:2. SDS-polyacrylamide gels (12%) showing the distribution of proteins in secretory (S), early maturation ( $M_E$ ) and midmaturation ( $M_M$ ) stage enamel at daily intervals (lanes 0-7) over 7 days of *in vitro* incubation.  
 Fig. 35:3. Enzymogram (non-reduced conditions) showing the distribution of endoproteinases (gelatinases) within untreated and TCA-treated secretory (S) and maturation ( $M_E + M_M$ ) stage enamel (E) and enamel organ cells (C) removed from the same locations on freeze-dried rat incisors (incubated at pH 8.0).

itates were redissolved in extraction buffer and these, and the untreated portions, were brought to a final concentration of 1.0-1.5  $\mu\text{g}$  protein/ $\mu\text{l}$  with sample buffer prepared without  $\beta$ -mercaptoethanol. One-hundred  $\mu\text{l}$  aliquots were loaded to the lanes of 5-15% linear gradient SDS-polyacrylamide gels prepared with 0.05% gelatin<sup>1,2</sup>, and the proteins were separated by electrophoresis. The SDS was removed by treating the gels for 1 hour in 2.5% Triton X-100<sup>5</sup>, and the gels were incubated for 3 days in Tris-HCl buffer, pH 8.0, supplemented with 10 mM  $\text{CaCl}_2$ <sup>2</sup>. The gels were soaked for 1 hour in 0.125% Coomassie Brilliant Blue R-250, then destained<sup>4</sup> (Fig. 35:3). Some TCA-treated samples were also incubated *in vitro* (Fig. 35:7).

## RESULTS

Enzymograms revealed numerous endoproteinases (gelatinases) within whole homogenates from enamel and enamel organs (Fig. 35:3). The molecular weights (MW) of proteinases extracted from the enamel organ were remarkably similar for both the secretory and maturation stages, and included strong activities near 62 and 66 kDa, and weaker activities near 41 and 90 kDa (doublet) and at the top of the gels (Fig. 35:3, lanes 3 & 4). In contrast, the MW of proteinases extracted from enamel samples were different between the secretory and maturation stages (Fig. 35:3). Secretory stage enamel showed two faint proteolytic bands near 38-40 kDa, a series of several more intense bands from 50-80 kDa, and other unresolved proteinases at the top of the gels (Fig. 35:3, lane 1). Maturation stage enamel showed one major band near 29



Figs. 35:4-6. SDS-polyacrylamide gels (12%) showing the distribution of proteins remaining in microfuge vials following 7 days of continuous *in vitro* incubation of secretory (S; Fig. 35:4), early maturation ( $M_E$ ; Fig. 35:5) and midmaturation ( $M_M$ ; Fig. 35:6) stage enamel under various conditions: A = pH 7.5 + 20°C; B = pH 7.5 + 37°C (control); C = pH 5.0; D = pH 9.0; E=boiled; F= + sample preparation buffer; G = + amidino-PMSF [27]; H = +PMSF [350]; I = +TLCK [50]; J = +TPCK [100]; K = + $\alpha$ -macroglobulin; L = +soybean trypsin-chymotrypsin inhibitor [20]; M = +bestatin [40]; N = +elastatinal [1]; O = +plasminogen; P=boil+plasminogen; Q = +enterokinase; R=boil +enterokinase; S = +aprotinin [50]; T = +leupeptin [30]; U = +PMSF [27]; V = +E-64 [10](in 35:4 V=PMSF [27]); W = +chymostatin [25]; X = +pepstatin A [25]; Y = +phosphoamidon [100]; Z = +benzamidine [825]. Asterisks, effective inhibitors; [ ] =  $\mu$ g/ml.

kDa and faint bands near 50 kDa as well as at the top of the gels (Fig. 35:3, lane 2). TCA altered the apparent MW of proteinases in enamel (Fig. 35:3, lanes 5 & 6). TCA-treated secretory stage enamel showed one very intense band near 50 kDa and other bands near 39, 46, 62 and 66 kDa (Fig. 35:3, lane 5). TCA-treated maturation stage enamel showed an intense band near 29 kDa, similar to the one seen in untreated controls, as well as other clear bands near 50, 62, and 66 kDa (Fig. 35:3, lane 6). TCA-treated enamel organs for the most part showed few differences from untreated controls except for some minor bands near 50 and 70 kDa (Fig. 35:3, lanes 7 & 8 vs. lanes 3 & 4).

As reported previously<sup>4</sup>, enamel samples incubated *in vitro* at 37°C and pH 7.5 in PBS-Triton buffer showed rapid breakdown of enamel proteins located near 30-32 kDa, and either slow or fast degradation of those from 18-28 kDa depending upon whether the samples were taken from the secretory (slow), early maturation (intermediate) or midmaturation (fast) stages of amelogenesis (Fig. 35:2). The *in vitro* degradation of enamel proteins was inhibited by TCA treatment (Fig. 35:7) and blocked completely by boiling the samples, or exposing them to PMSF (0.1-2 mM) prior to incubation, or, by incubating them at 37°C in the presence of SDS and  $\beta$ -mercaptoethanol (Fig. 35:4-6). Aprotinin and trypsin-chymotrypsin inhibitor effectively blocked degradation of enamel proteins in samples from the maturation stage but not those from the secretory stage (Fig. 35:4-6). In contrast, acidic (pH 5) or alkaline (pH 9) conditions, and

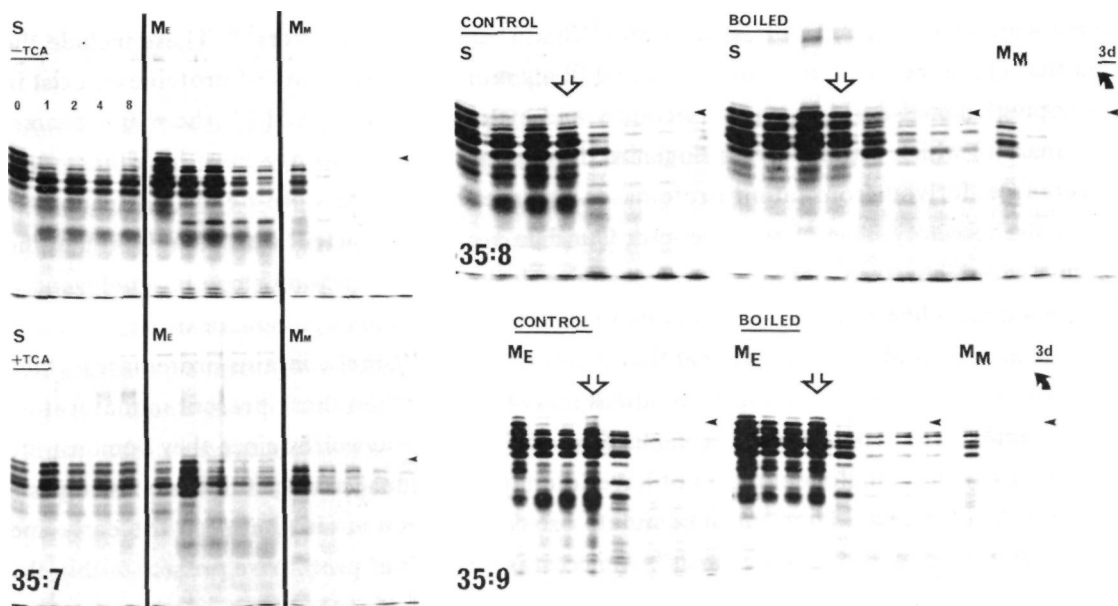


Fig. 35:7. SDS-polyacrylamide gels (12%) showing the distribution of proteins in untreated (top) and TCA-treated (bottom) secretory (S), early maturation (M<sub>E</sub>) and midmaturation (M<sub>M</sub>) stage enamel samples at 1, 2, 4, and 8 days of continuous *in vitro* incubation. Arrowheads = 30 kDa (also in Figs. 35:8 and 35:9).

Fig. 35:8. SDS-polyacrylamide gels (12%) showing the distribution of proteins in unboiled (control) and boiled secretory stage enamel at daily intervals over 7 days of continuous *in vitro* incubation. On the third day (arrows), fluids from unboiled midmaturation stage enamel samples (M<sub>M</sub>) were added to the secretory stage samples.

Fig. 35:9. SDS-polyacrylamide gels (12%) showing the distribution of proteins in unboiled (control) and boiled early maturation stage enamel at daily intervals over 7 days of continuous *in vitro* incubation. On the third day (arrows), fluids from unboiled midmaturation stage enamel samples (M<sub>M</sub>) were added to the early maturation stage samples.

chymostatin (pH 7.5), partially inhibited degradation of enamel proteins from the secretory and early maturation stages but not those from the midmaturation stage (Fig. 35:4-6). Other proteinase inhibitors including benzamidine, TLCK, TPCK, E-64,  $\alpha$ -macroglobulin, bestatin, elastatinal, phosphoamidon, pepstatin A, and leupeptin showed little or no inhibitory effect on *in vitro* degradation of enamel proteins (Fig. 35:4-6). Experiments done with boiled and unboiled secretory and early maturation stage enamel samples mixed with unboiled midmaturation stage enamel samples (Figs. 35:8-9) revealed that the pattern and rate of breakdown of enamel proteins in this material was typical of the stage from which the proteinase was taken (M<sub>M</sub>) and not of the stage from which the enamel proteins were derived (S or M<sub>E</sub>).

## DISCUSSION

The results of this study document the importance of knowing precisely the stage of amelogenesis from which enamel samples are derived, since the hydrolytic properties and MW of constituent proteinases change over time, and the importance of knowing the types of endoproteinases contained within enamel organ cells since they can produce a contamination of the enamel samples through wiping. This study also provides a simple demonstration of how slight differences in preparative technique, such as exposure to TCA, can alter dramatically the apparent MW and behavior of proteinases present within an enamel sample.

The findings of this study are consistent with two concepts that have emerged from the

recent work by Overall<sup>1</sup>, DenBesten<sup>2</sup>, Cater<sup>3</sup>, Smith<sup>4</sup> and their coworkers<sup>1-4</sup>. These include the idea that (1) more than one proteinase, and likely more than one class of proteinase, exist in developing enamel during both the secretory and maturation stages, and (2) the major enamel proteinase(s) which degrade(s) amelogenins during the maturation stage must be either newly secreted or derived from latent proteinases or proenzymes that were deposited in enamel during the secretory stage of amelogenesis. Our data are also consistent with the notion that the enzyme with highest activity against amelogenins during the maturation stage of amelogenesis may be a trypsin-like serine proteinase (about 29 kDa) having a pH optimum near pH 8.0<sup>3</sup>.

The *in vitro* studies further reveal that secretory stage enamel contains proteinases which are capable of degrading amelogenins, albeit less efficiently than those present in maturation stage enamel<sup>4</sup>. The cross zone experiments (Fig. 35:8-9) are noteworthy since they demonstrate unequivocally that amelogenins do not have to undergo gradual extracellular processing as a prerequisite to the rapid and more complete breakdown seen *in vivo* between the early and midmaturation stages of amelogenesis<sup>4</sup>. Hence, it is the type of proteinase present within the developing enamel, and its absolute hydrolytic activity, which seem most important to the maturation phenomenon (rapid loss of amelogenins). While the proteinases responsible for degrading amelogenins in secretory stage enamel remain to be characterized, our data suggest that some of the key enzymes are also serine proteinases with biochemical properties which are distinct from those found in maturation stage enamel. The enzymograms (Fig. 35:3) also suggest that some secretory stage enamel proteinases may exist naturally as aggregates while others may constitute latent activities. There also appears to be at least two groups of proteinases near 62 and 66 kDa, a pair of very active proteinases, perhaps metalloproteinases<sup>1</sup>, associated with the enamel organ and/or labial connective tissues, and a another pair of latent proteinases within secretory and maturation stage enamel which may not be metalloproteinases.

The finding that aprotinin acts as a more potent inhibitor during the maturation stage than during the secretory stage suggests there are specific bonds within the amelogenin molecule which are being cleaved by the maturation stage enamel proteinases<sup>1-4</sup>. It is well known that the amino acid sequence from residues 15-18 at the reactive site of aprotinin is -Lys-Ala-Arg-Ile-<sup>6</sup>. Since there are no known -Lys-Ala- combinations in amelogenin<sup>7</sup>, we suspect aprotinin effectively inhibits amelogenin degradation during the maturation stage because it interferes with hydrolysis of the -Arg-His- bond at residues 31 and 32 where there is the sequence -Met-Ile-Arg-His-<sup>7</sup>. This interpretation provides additional support for the concept that some enamel proteinases are relatively specific for amelogenins. (*Supported by the MRC of Canada*)

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## Poster 35

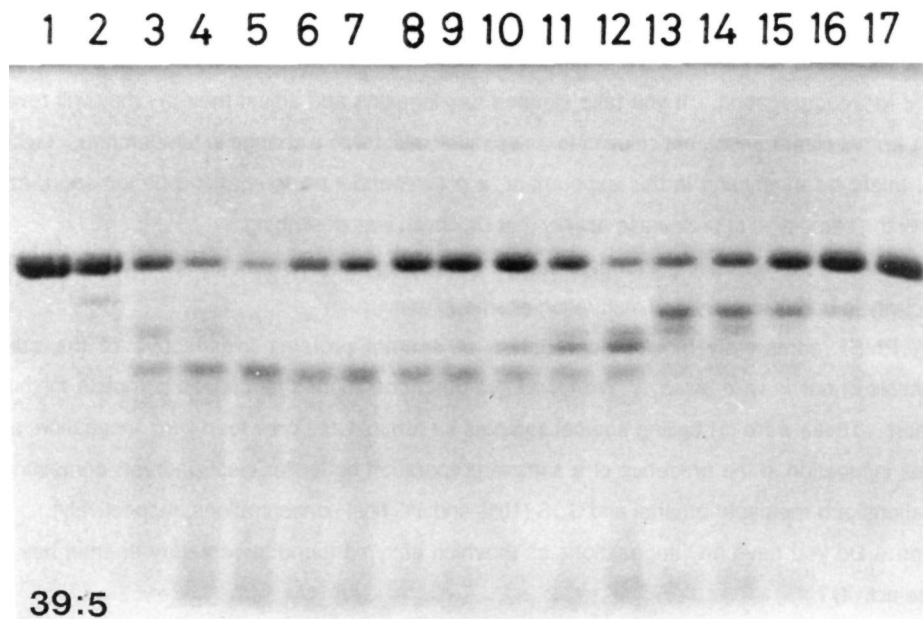
Moreno: Dr. Smith, your observation about the greater efficiency of the enzyme in breaking down the amelogenins, could that indicate that with time the original matrix undergoes changes so that it becomes more easily accessible to enzymatic action.

Smith: That is an issue which we were trying to address in the experiments (Figs 35:8 and 35:9), where we took enamel proteins from the secretory stage and mixed them with enamel proteinases derived from the mid-maturation stage. The results suggest it is not necessary for the enzymes in the mid-maturation stage to have pre-processed protein for them to act efficiently on the protein..

Moreno: Then what is the alternative explanation, Charlie?

Smith Well it just seems this system is geared to activate or bio-synthesise de novo, enzymes that are very efficient at degrading amelogenins.

Tanabe: Dr. Smith, I am interested in the degradation of enamel proteins, and the function of proteolytic enzymes in enamel. In fact we found a serine protease-like enzyme in the secretory stage of porcine enamel, which we investigated using 20 kDa amelogenin, and we found the enzyme degraded the amelogenin between His and Ala, (Tanabe, T. Tsurumi Shigaku, 10:443-452, 1984). Fig.39:5 illustrates the effects of pH on the mode of enzyme action against 20kDa amelogenin. On the left side the reaction solution is acidic, and on the right alkaline. The proteolytic enzyme and 20kDa amelogenin were extracted from porcine immature enamel and purified. The substrate (0.01 ml of 0.5 % 20kDa amelogenin) was mixed with 0.1 ml of buffer solution and then 0.02 ml of the enzyme was added. The mixture was incubated at 37C. for 24 hrs and stopped by adding SDS sampling solution.



The samples were electrophoresed on 15 % polyacrylamide gel containing SDS. The gel was stained with CBB. The activity of the enzyme was tested in the range of pH 4 - 11, and the buffers used were : 0.05 M acetate buffer (column No.1- 6), 0.05 M tris buffer (column No.7 -12) and 0.05 M carbonate buffer (column No.13 -17). The final pH of each reaction mixture was , 1: pH 4.1, 2: pH 4.6, 3: pH 5.1, 4: pH 5.7, 5: pH 6.1, 6: pH 6.5, 7: pH 6.9, 8: pH 7.2, 9: pH 7.6, 10: pH 8.0, 11: pH 8.5, 12: pH 9.0, 13: pH 9.0, 14: pH 9.5, 15: pH 10.0, 16: pH 10.5 and 17: pH 11.0. You can see that there are several different products in relation to the different pH levels. The N-terminal of the main product of column 5, at pH 6, was Ala. We feel therefore, that the function of this enzyme is dependant on the pH of the environment. Would you comment on this please?

Smith: I would like to compliment you on a nice experiment. The data in our poster does not disagree with anything you just showed, if I understood correctly, this is secretory stage enamel. For example, we found that degradation of enamel proteins was inhibited at both pH 5.0 and pH 9.0 in secretory stage samples, whereas pH seemed to have little or no effect on protein degradation in mid-maturation stage enamel samples. This difference could be explained by proposing, as several other workers already have, that there is a multi-enzyme system operating in developing enamel, which expresses different hydrolytic potential depending upon the stage of amelogenesis. Similarly, the enamel proteinases may be organised functionally in a cascade. The problem with inhibitors is that we cannot easily distinguish blocking action on a proteinase toward the bottom of the cascade, which actually hydrolyses the amelogenins, from blocking action on a different proteinase toward the top of the cascade which may be required for activating the other proteinase. As well we can isolate a proteinase from the developing enamel, but how it behaves in "purified" form may not necessarily be representative of it's properties *in situ* where these enzymes may be interacting in a co-ordinated manner like a symphony.

Snead: I had just a comment on Dr. Tanabe's data based upon something that Dr Fincham once showed from Dr. N. Simmon's work in (U.C.L.A.), that the amelogenins from the secretory stage are extremely pH dependent for reaggregation. If you take isolated amelogenins and adjust their pH they will reversibly aggregate from a clear transparent solution to an opaque colloid with a change in temperature. I wonder if what you might be examining in this experiment, a pH dependent aggregation phenomenon, and not necessarily the same type of proteinase activity that Dr. Smith was describing.

DenBesten: I may be misreading your data but are you saying that phenylmethylsulfonyl fluoride (PMSF) inhibits activity in both secretory and maturation enamel?

Smith PMSF completely blocks degradation of enamel proteins irrespective of the stage of amelogenesis in our *in vitro* assay. We found two other conditions that caused complete inhibition of degradation. These were (1) boiling enamel samples for ten minutes prior to *in vitro* incubation, and (2) continuous incubation in the presence of a sample preparation buffer for electrophoresis containing high concentrations of b-mercapto-ethanol and SDS (10% and 1% final concentrations, respectively).

DenBesten: Do you have any suggestions as to which enzyme found in secretory enamel has serine proteinase activity?

Smith: The incubations with PMSF were done in the presence of Calcium ions since the whole enamel homogenates were used. I cannot identify which proteinase(s), in the enzymogram (Fig. 35:3), might

correspond to the PMSF-sensitive activity. I am under the impression that Dr. Sasaki had isolated a serine proteinase from enamel.

Sasaki, S: We have not carefully examined the PMSF-sensitivity of the enzyme yet. So, I cannot say whether it is or not.

McKee: Do you have any evidence that the protein material secreted in the early maturation zone are proteinases?

Smith: We have not been able to find any convincing evidence in our studies with radiolabeled methionine, that some of these newly formed proteins correspond to proteinases. However, methionine may not be the best label for enamel proteinases, and more work is needed to clarify this question.

McKee: Can you do an enzymogram-fluorograph overlay to determine if newly synthesised protein has enzymatic activity?

Smith: I think the problem is to find a good radioactive label that will allow newly synthesised enzymes to be distinguished from "older" ones that may have been deposited in the enamel during the secretory stage of amelogenesis and later activated during the maturation stage.

McKee: But would you not be able to determine if a certain molecular weight protein was newly synthesised and that it had enzymatic activity using  $^{35}\text{S}$  or  $^3\text{H}$  methionine for example. Wouldn't that partly answer the question?

Smith: We have attempted to do enzymograms with enamel samples taken from rats injected with  $^{35}\text{S}$ -methionine. We got no convincing evidence of a correlation between the digestion bands, for example, near 30 kDa in the enzymograms and obvious labelled bands in companion fluorographs in material at 1 hour after injection of the radioactive methionine.