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# Note

## Determination of proline iminopeptidase activity in bovine dental germs by high-performance liquid chromatography

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In the course of tooth formation, special cells such as ameloblasts or enamel-forming cells and odontoblasts or dentine-forming cells differentiate in the tooth germ. They synthesize and secrete the special proteins needed in order to make calcified tissues, such as enamel and dentine. During this calcification process, proteolytic enzyme activities are detectable in enamel [1] and in dentine [2].

The amino acid composition of collagens [3] and of amelogenins [4,5], which are produced from different cells, are characterized by a high concentration of proline residues. For the complete degradation of these proline-rich proteins, proline-specific endo- and exopeptidases must attack the peptides after specific proteolysis has taken place. In the maturation step of enamel formation, a reduction in the enamel proteins, amelogenins, has been demonstrated [6].

Earlier we determined proline-specific exopeptidases by high-performance liquid chromatography (HPLC) [7]. This paper describes a new method for the determination of proline iminopeptidase (EC 3.4.11.5) activity in dental germs, using Pro-Gly-Gly as the substrate.

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## EXPERIMENTAL

### *Chemicals*

L-Pro-Gly-Gly, Gly-Gly and L-Pro-Gly were obtained from Sigma (St. Louis, MO, U.S.A.).

### *Preparation of bovine dental tissues for enzyme source*

Fresh bovine dental tissues were obtained packed in ice from the slaughterhouse. Dental sac and pulp were carefully separated from the unerupted lower incisors. They were kept frozen at −20°C until used.

The sacs and pulps were rinsed in cold saline, blotted and homogenized in 7 volumes of 0.25 *M* sucrose with an Ultra-Turrax homogenizer, and the homogenate was then centrifuged for 10 min at 600 *g* to remove tissue debris. Protein concentrations were determined by the method of Hartree [8] with bovine serum albumin as standard.

### *Chromatographic determination of proline iminopeptidase activity*

Proline iminopeptidase (EC 3.4.11.5) specifically hydrolyses the N-terminal proline residue from peptides. With Pro-Gly-Gly as the substrate, the formation of Gly-Gly and the decrease of the concentration of Pro-Gly-Gly were determined by HPLC [7]. The assay medium (500 μl) contained 30.0 μmol of Tris–HCl buffer (pH 8.0), 0.70 μmol of Pro-Gly-Gly, 1.0 μmol of manganese chloride, 20–100 μg of tissue homogenate and water. Four different assay mixtures with different homogenates were incubated at 37°C for 30 min, and the reactions were stopped by the addition of 400 μl of 10% perchloric acid to each tube. After centrifugation of the assay tubes at 3000 *g* for 10 min, the concentration of the peptides in the clear medium solution was determined by HPLC. The injection volume was 10 μl.

Enzymatically formed Gly-Gly from the Pro-Gly-Gly in the assay mixture was separated by HPLC with a mobile phase composed of 10.0 m*M* potassium dihydrogenphosphate buffer (pH 2.1) containing 1.0 m*M* 1-octanesulphonate, and the eluate was monitored for peptides at 210 nm. The column (150 mm × 4.6 mm I.D.) was packed with Zorbax ODS, particle size 5 μm, supplied by Dupont (Wilmington, DE, U.S.A.). The guard column (50 mm × 4.0 mm I.D.) containing Parmaphase ETH, particle size 30 μm (Dupont), was connected to the analytical column.

### *Activity assay of other enzymes*

Dipeptidyl peptidase IV (EC 3.4.14.5) and aminopeptidase P (EC 3.4.11.9) activities were also determined according to previously reported procedures [7,9].

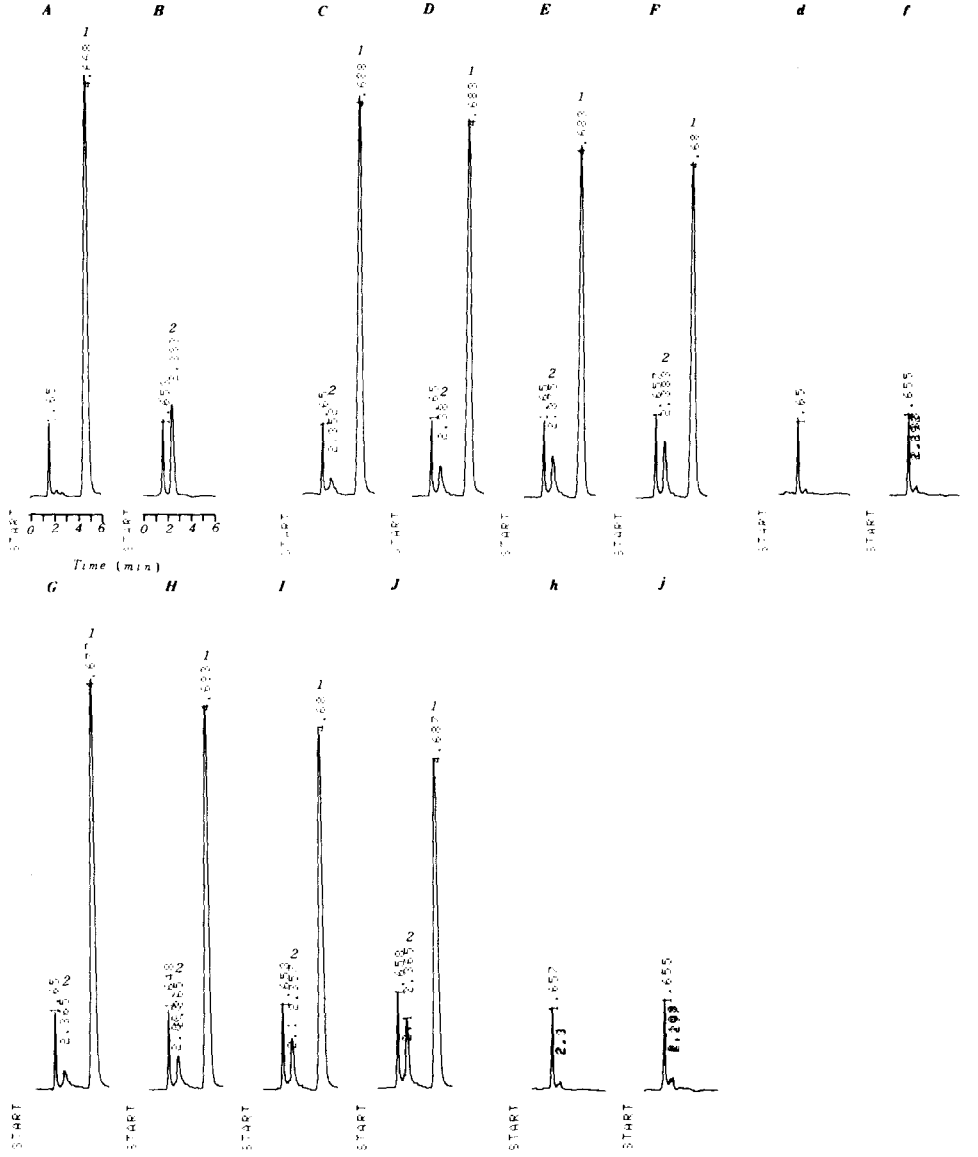


Fig. 1. Typical chromatographic patterns of the hydrolysis of Pro-Gly-Gly with different concentration of bovine dental sac homogenate (C, 16.8 $\mu$g; D and d, 33.6 $\mu$g; E, 50.4 $\mu$g; F and f, 67.2 $\mu$g) and bovine dental pulp homogenate (G, 22.0 $\mu$g; H and h, 44.0 $\mu$g; I, 66.0 $\mu$g; J and j, 88.0 $\mu$g). Peak 1 (A), Pro-Gly-Gly (7.8 nmol, retention time 4.67–4.68 min), decreased in C, D, E, F, G, H, I and J, and peak 2 (B), Gly-Gly (2.8 nmol, retention time 2.37–2.38 min), increased at each protein concentration (C, D, E, F, G, H, I and J). Traces d, f, h, and j indicate respective controls of D, F, H, and J without substrate. Mobile phase, 10.0 m*M* potassium dihydrogenphosphate (pH 2.1), containing 1.0 m*M* 1-octanesulphonate. The chromatograms were recorded with a Shimadzu C-R3A Chromatopac graphic data system, which monitored the absorbance (0.04 a.u.f.s) of peptides at 210 nm. The numbers beside the peaks indicate retention times (min).

RESULTS AND DISCUSSION

The retention times of Pro-Gly-Gly and Gly-Gly were 4.7 and 2.4 min, respectively, with the mobile phase described.

Incubation of Pro-Gly-Gly with an increasing amount of bovine dental sac homogenate resulted in a gradual decrease in the Pro-Gly-Gly peak with a concomitant increase in the 2.4-min peak corresponding to Gly-Gly (Fig. 1). The amount of Pro-Gly-Gly degraded and amount of Gly-Gly produced were plotted against the amount of homogenate protein used (Fig. 2). The former two were stoichiometrically related to each other. Pro-Gly, which has a retention time of 7.9 min, was not detected in the incubation mixture.

From these observations the presence of proline iminopeptidase activity in bovine dental sac and pulp is evident, since proline iminopeptidase is an aminopeptidase that can release an N-terminal proline residue from peptides of any length: Pro-X-Y-. The specific activity of the enzyme from bovine sac and pulp was calculated to be $67.1 \pm 4.4$ and $55.1 \pm 2.8$ nmol $min^{-1}$ (mg protein)$^{-1}$ (mean $\pm$ S.D. for four different determinations), respectively.

Purified proline iminopeptidase from pig kidney [10] and bovine kidney [11] showed the highest substrate specificity for Pro-Leu-Gly-$NH_2$ (melanocyte-stimulating hormone release-inhibiting factor) rather than for Pro-Gly-Gly. If this is also the case for the bovine sac enzyme, we can assume that the bovine sac homogenate exhibits about half or one third of the kidney activity.

Other enzymes in bovine dental pulp that attack proline-containing pep-

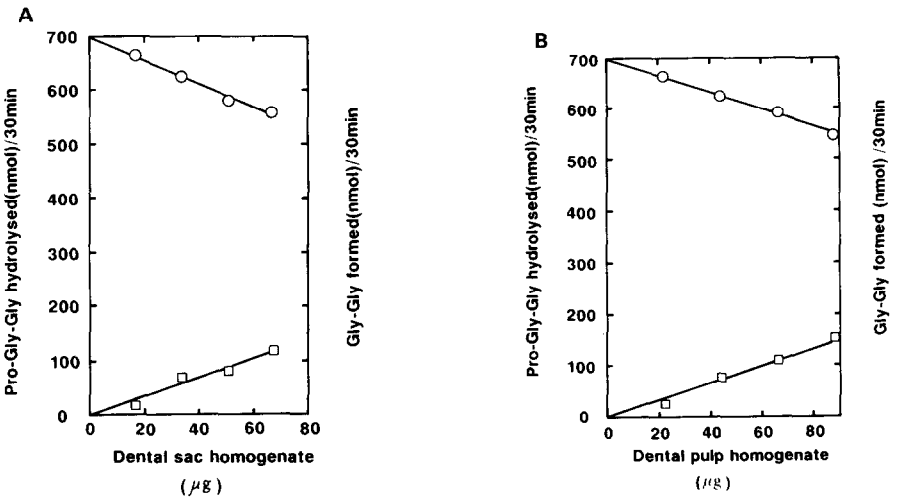


Fig. 2. Dependence of Gly-Gly formation on dental sac (A) and pulp (B) homogenate concentration. Gly-Gly was formed from Pro-Gly-Gly by proline iminopeptidase activity. Hydrolysis of Pro-Gly-Gly (○) and formation of Gly-Gly (□) were stoichiometrically related under the analytical conditions described in Experimental.

tides, i.e. dipeptidyl peptidase IV [1.4–1.9 nmol $min^{-1}$ (mg protein)$^{-1}$] and aminopeptidase P (quite low, not estimated), were also assayed, but their activities were lower than the activity of proline iminopeptidase.

In view of the proline iminopeptidase activity in the bovine dental germ, it is obvious that proline-rich proteins such as collagens and amelogenins would be further degraded after they undergo initial proteolysis [12,13]. Together with other peptidase such as prolidase and prolinase, proline iminopeptidase would cleave the peptides into their individual amino acids.

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