PROTEINS IN EMBRYONIC BOVINE ENAMEL

BY

Ikuo KANEKO*

ABSTRACT

The fractionation and the amino acid composition of the organic matrix of embryonic bovine enamel was studied. EDTA- and water-soluble fraction dissolved in phosphate buffer showed a temperature-dependent properties; at low temperatures it formed a clear solution, but at room temperature heavy precipitations occurred. This phenomenon was reversible.

EDTA- and water-soluble fraction prepared in mild conditions was first chromatographed on Sephadex G-25 column. Four fractions were obtained. The main fraction comprised nearly half of the material and its molecular weight was found by gel filtration to be greater than 3,500. The temperature-dependent phenomenon above stated was observed only in the main fraction. The result of the fractionation with Sephadex G-100 revealed that a portion of the main fraction had molecular weight greater than 100,000.

The results of the amino acid analysis showed that the EDTA- and water-soluble fraction was a high proline component. On the other hand, the EDTA-insoluble fraction was a high proline-glutamic acid-histidine component.

The EDTA-insoluble fraction was extracted with organic solvents and water. About 73% was solubilized, and the amino acid composition of the extracted residue relatively changed from the mother fraction; the residual protein contained only small amount of histidine. These results were discussed with reference to enamel maturation.

INTRODUCTION

Several investigators studied the amino acid composition of embryonic bovine enamel protein (Glimcher et al. 1961, Burgess and MacLaren 1965, Glimcher et al. 1964) and the results of the latter two reports showed great similarity. The general features were a very high proline content, accounting for a quarter of the total residues. The content of glutamic acid, leucine and histidine was also high. Hydroxyproline, hydroxylysine and cystine were either absent or very little.

Embryonic bovine enamel protein was suggested to be constituted of several species of proteins by some investigators in recent years. Bonar (1966) studied the neutral soluble enamel protein by gel filtration and ultracentrifuge and found that the protein was a concentration-dependent aggregating system of proteins. Nikiforuk and Simmons (1966) stated by turbidimetry

* 金子育夫: Department of Biochemistry (Chief: Prof. S. ARAYA), School of Dentistry, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku).
Received for publication, September 26, 1966.

that the embryonic bovine enamel protein showed a temperature-dependent properties. Burgess and MacLaren (1965) tried to fractionate EDTA-soluble enamel protein by gel filtration and electrophoresis. The present paper mainly deals with a further study of the fractionation of EDTA- and water-soluble embryonic bovine enamel protein. To prevent hydrolysis of the protein, the sample was treated around neutral conditions throughout the experiment. The amino acid compositions of the EDTA- and water-soluble and EDTA-insoluble component were also studied. EDTA-insoluble material was extracted with several organic solvents and water, and the amino acid analysis of the residual protein was undertaken in order to know some aspects of protein loss during enamel maturation.

Materials and Methods

Tissue source. Jaws of young bovines were obtained from a slaughter house and stored at −15°C. Embryonic teeth were separated from the jaws, and surrounding soft tissues and the pulpal tissues were removed. The remaining soft tissues on the coronal part of the teeth were removed with gauge while rinsing the teeth with distilled water. Teeth which were heavily stained with blood were discarded. The teeth were washed with distilled water several times and blotted dry. Cheese-like enamel was obtained from the cervical portions of the labiale surface with blunt spatula. Care was taken not to scratch dentine. Pooled enamel was dried overnight at 50°C and then pulverized. About 100 teeth were used in the experiment.

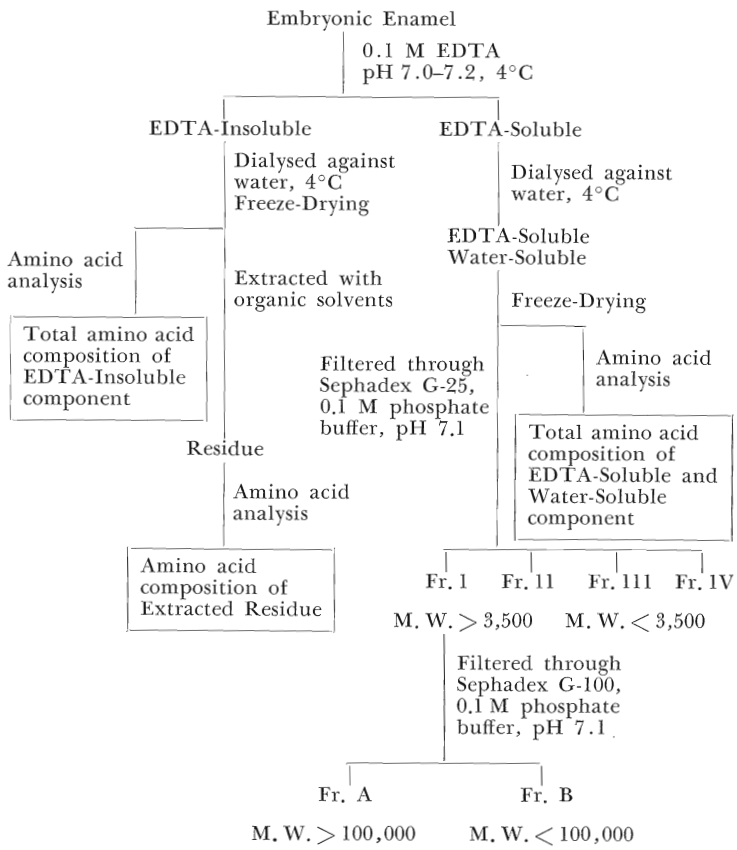
Isolation of the organic matrix of enamel (Scheme 1.). The enamel powder was decalcified with 0.1 M EDTA (pH 7.0–7.2) in a dialysis tube at 4°C. The decalcifying solution was stirred over a magnetic stirrer, and was changed every two days. When the decalcification completed, the EDTA-insoluble component was separated by centrifugation at low temperatures.

EDTA-insoluble fraction. The fraction was dialysed against distilled water at 4°C and freeze-dried. A portion of the material was subjected to amino acid analysis (Total amino acid composition of EDTA-Insoluble component) and the rest of the sample was successively extracted with acetone, ether, chloroform–methanol (1 : 3, v/v) for two days at room temperature respectively. The residue was then extracted with pyridine at 60°C for 24 hours. The remaining insoluble material was extracted with distilled water extensively at room temperature. The filtrate was freeze-dried and the residue was dried in a vacuum desiccator over P_2O_5 and the amino acids were analyzed (Amino acid composition of Extracted Residue).

EDTA-soluble fraction. The fraction was dialysed exaustively against distilled water (toluene was added for bacteriostasis) at 4°C to remove EDTA. A small amount of precipitation occurred. It was separated by

centrifugation. Each fraction was freeze-dried. A portion of the EDTA- and water-soluble fraction was subjected to amino acid analysis (Total amino acid composition of EDTA- and Water-Soluble component). The rest of the material was dissolved in 0.1 M phosphate buffer containing 0.1 M NaCl (pH 7.1, ionic strength=1.3) at 4°C. And a fractionation was carried out by gel filtration.

Gel filtration. Sephadex G-25 column (2.5 cm × 60 cm) and G-100 column (2.5 cm × 60 cm) were equilibrated with 0.1 M phosphate buffer containing 0.1 M NaCl at 4°C. The EDTA- and water-soluble fraction prepared was first eluted through the Sephadex G-25 column with the phosphate buffer. Four fractions were obtained (Fig. 1). The major fraction was pooled, concentrated by ultrafiltration, and then filtered through the Sephadex G-100 column (Fig. 2). The elutions were carried out at low temperatures using the same phosphate buffer. Optical density at 253 mμ was successively recorded (UVI CORD, LKB).



Scheme 1. Fractionation of embryonic bovine enamel

Table 1. Amino acid compositions of embryonic bovine enamel
(Values are expressed as residues/1,000 total residues)

	EDTA-Sol. Water-Sol.	EDTA-Insoluble		Burgess et al.* (1965)
		Total	Extracted Residue	
Aspartic acid	16	13	36	34
Threonine	34	23	30	28
Serine	40	49	76	45
Glutamic acid	73	152	172	145
Proline	241	177	179	250
Glycine	54	66	89	54
Alanine	17	15	20	22
Cystine	0	2	4	0
Valine	66	39	44	41
Methionine	47	52	48	54
Isoleucine	65	28	27	35
Leucine	85	85	112	96
Phenylalanine	16	24	25	27
Tyrosine	52	36	23	49
Hydroxyproline	0	0	0	3
Hydroxylysine	0	0	0	5
Lysine	68	35	23	16
Histidine	81	132	52	65
Arginine	44	56	39	19
Total	999	1001	1002	988

* Undemineralized samples. Stages of complete matrix.

Amino acid analysis and nitrogen determination. EDTA- and water-soluble fraction, EDTA-insoluble fraction and its extracted residue were analyzed for their amino acid compositions. The samples were hydrolyzed under vacuum in sealed tubes with double distilled constant-boiling HCl for 24 hours at 110°C. An automatic amino acid analyzer was used for the analysis. Nitrogen was determined by micro-Kjeldahl method.

Results

Enamel powder used for the experiment was 4.55 g. After decalcification with 0.1 M EDTA, 600 mg (13.1%) of EDTA-soluble fraction and 315 mg (6.9%) of EDTA-insoluble fraction were obtained. Accordingly, the total organic matrix content of the embryonic enamel used for the experiment was 20% per dry enamel. EDTA-soluble fraction was separated into two subfractions owing to the difference of solubility in water; EDTA- and water-soluble fraction (10.5%) and EDTA-soluble and water-insoluble frac-

tion (2.6%). The latter fraction contained 86.3% protein on N basis.

Table 1 summarized the amino acid compositions of EDTA- and water-soluble fraction, EDTA-insoluble fraction (total) and the extracted residue. The protein content of each fraction was calculated to be 38%, 57.2% and 35.2% (excluding tryptophan) respectively. The amino acid composition of EDTA- and water-soluble fraction showed a very high proline content, a quarter of the total residues. The concentration of leucine, histidine and glutamic acid was relatively high. Hydroxyproline and hydroxylysine and cystine were not detected. EDTA-insoluble fraction, on the other hand, had a high content of glutamic acid and histidine as well as proline. Total content of the three amino acids comprised nearly half of the total residues. Both hydroxyproline and hydroxylysine were not detected.

When EDTA-insoluble fraction was extracted with water after the treatment with some organic solvents, some portions (43.6% by weight) dissolved. The solubilized portion contained 87.2% protein on N basis. General characteristics of the amino acid composition of the extracted residue was a decrease of basic amino acid content and a slight increase of acidic components. The content of serine, leucine and glycine was also increased. The decrease of histidine was most significant.

Extraction with water of EDTA-insoluble fraction after the treatment with some organic solvents resulted in the solubilization of some portions (43.6% by weight) of the fraction. The solubilized portion contained 87.2% protein on N basis. General characteristics of the amino acid composition of the extracted residue was a decrease of basic amino acid content and a slight increase of acidic components. The content of serine, leucine and glycine was also increased. The decrease of histidine was most significant.

A temperature-dependent phenomenon was observed in EDTA- and water-soluble fraction. When the freeze-dried material was dissolved in the phosphate buffer above mentioned, it formed a clear solution at 4°C. But at room temperature, heavy precipitations occurred in the solution. When the sample was brought to low temperatures the precipitations redissolved. Thus the phenomenon was reversible.

Figure 1 indicated the elution pattern of the EDTA- and water-soluble fraction by Sephadex G-25 column. One main fraction and subsequent three fractions were obtained. Fraction 1 was excluded from the column, and its molecular weight was greater than 3,500. Relative concentration of Fraction 1 was 44.5% in terms of optical density at 253 mμ. Subsequent minor fractions were retained in the column, molecular weight of which were less than 3,500. Only the tubes corresponding to the Fraction 1 showed the temperature-dependent aggregating properties.

Fraction 1 was filtered through Sephadex G-100 column (Fig. 2). A

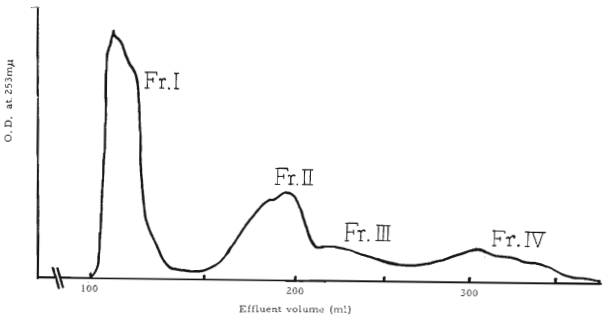


Fig. 1. Fractionation of EDTA-and Water-Soluble Component of Young Enamel. Sephadex G-25.

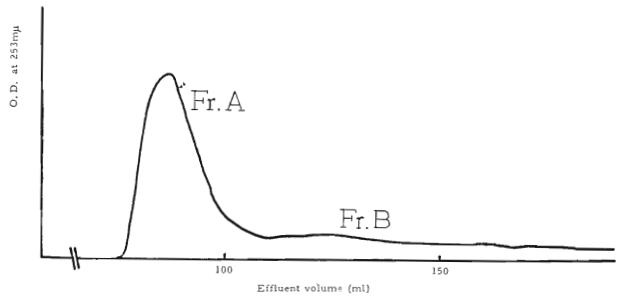


Fig. 2. Elution Pattern of Fr. I through a Column of Sephadex G-100

main peak whose molecular weight was greater than 100,000 and a broad one were obtained. Because of a small amount of the material recovered, no further experiment was carried out in this investigation.

Discussion

The embryonic teeth were obtained from 12-month old or younger bovines and the teeth whose crown formation had not completed or the teeth whose root formation had just begun were used for the experiment. Since hydroxyproline and hydroxylysine were not detected in the present experiment, it would be safe to conclude that no coronal cementum had not been formed on cheese-like enamel.

Nearly half of the EDTA-insoluble fraction became extracted with water after treatments with several organic solvents and some changes of amino acid composition including a significant decrease of histidine were observed. Burgess and MacLaren (1965) reported that at least 80% of amino acids was lost during enamel maturation and also reported that there

were much differences of amino acid composition between embryonic enamel and mature enamel. Among the differences, increase of the content of glycine and serine and a decrease of histidine in mature enamel were observed. As low histidine content in EDTA-insoluble component of mature enamel was also reported (Glimcher and Levine 1966), the extraction procedure in this experiment might reflect some changes in protein loss during enamel maturation.

The results of fractionations by gel filtration revealed that there were at least five species of proteins (peptides) in embryonic enamel matrix. Their molecular weights were wide-ranged and unexpectedly, over 50% of the proteins (peptides) had molecular weights less than 3,500. A fractionation of EDTA-soluble component of developing enamel protein was also undertaken (Burgess and MacLaren 1965) with Sephadex G-50. But the elution pattern was not similar to those of the present paper. The disimilarity might be due to differences of the samples employed. Glimcher and Levine (1966) reported that proteins whose molecular weight were greater than 3,500 existed only 10% of the total weight in mature enamel (EDTA-soluble fraction). Since over 80% of embryonic enamel protein was reported to be lost during maturation, the process would involve degradation of some protein components in EDTA-soluble fraction. Accordingly the embryonic peptides whose molecular weight were less than 3,500 seemed to be in degradating process.

The temperature-dependent phenomenon was also reported by Nikiforuk and Simmons (1966). High proline content in enamel matrix was suggested to be the cause of the phenomenon. And it was also indicated to be analogous to a temperature-dependent aggregation of poly-L-proline solution. But casein which also has high content of proline and glutamic acid has been reported to undergo temperature-dependent aggregation (Reithel 1963). In this case, Ca ion plays an important role in stabilizing the solution or micelles. It is not known whether Ca ion provides some effects on the temperature-dependent phenomenon observed in enamel protein solution, further study on casein seems desirable.

References

1) Bonar, L. C.: The structure of the protein components in developing enamel. J. D. Res., **44**: 1116, 1966.
2) Burgess, R. C. and MacLaren, C. M.: Proteins in developing bovine enamel. 'Tooth Enamel' p. 74, 1965.
3) Glimcher, M. J., Mechanic, G., Bonar, L. C. and Daniel, E. J.: The amino acid composition of the organic matrix of decalcified fetal bovine dental enamel. J. Biol. Chem., **236**: 210, 1961.
4) Glimcher, M. J., Mechanic, G. L. and Friberg, U. A.: The amino acid composition

of the organic matrix and the neutral-soluble and acid-soluble components of embryonic bovine enamel. Biochem. J., **93**: 198, 1964.
5) Glimcher, M. J. and Levine, P. T.: Studies of the proteins, peptides and free amino acids of mature bovine enamel. Biochem. J., **98**: 742, 1966.
6) Nikiforuk, G. and Simmons, N. S.: Purification and properties of protein from embryonic bovine enamel. J. D. Res., **44**: 1119, 1966.
7) Reithel, F. J.: The dissociation and association of protein structures. 'Advances in Protein Chemistry' **18**: 124, 1963.