Relative molecular mass determination of a major, highest relative molecular mass extracellular amelogenin of developing bovine enamel

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Proteins of developing bovine enamel were fractionated by molecular sieving and ion-exchange chromatography. The major fraction corresponding to the highest M_r amelogenin of M_r ~26000–30000 was isolated and its M_r determined by SDS-PAGE, molecular sieving on G-100 resin and high performance liquid chromatography and by sedimentation-equilibrium ultracentrifugation, the latter three procedures in guanidine hydrochloride. SDS-PAGE and HPLC molecular sieving, employing commonly used M_r standards, gave M_r values of ~22000–26000. SDS-PAGE and HPLC molecular sieving, using proline-rich CNBr peptides of collagen as standards, and sedimentation-equilibrium ultracentrifugation, gave M_r values of ~15000–18000 and ~17385, respectively. These latter values correspond well with those reported earlier and with the M_r of the major amelogenin computed from recent amino acid sequence data (~19000). It is concluded that the recently described, highest M_r amelogenin of M_r = 26000–30000 is not a new component but is identical to the proline-rich components having relative molecular masses ranging from 15000 to 18000 described much earlier by several groups of workers.

Relative molecular mass *Embryonic enamel* *Amelogenin precursor*

1. INTRODUCTION

The proteins of developing tooth enamel have been shown to consist of a very large number of components, most of which are rich in proline, glutamic acid, leucine and histidine (amelogenins) [1–5]. In original studies, the relative molecular masses of these components were found to be 15000–17000 [5,6]. More recently, however, Termine et al. [7] and Fincham et al. [8] have reported the isolation of what they considered to be a new, not previously reported proline-rich component, the major, highest M_r amelogenin, with an approximate M_r ~26000–30000. The purpose of the present experiments was to isolate this major, highest relative molecular mass amelogenin and to examine its relationship to the previously described species of M_r 15000–17000 having essentially the same amino acid composition.

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Abbreviations: Ser(P), *O*-phosphoserine; Gdn-HCl, guanidine hydrochloride; HPLC, high performance liquid chromatography

2. MATERIALS AND METHODS

2.1. *Preparation of enamel*

The immature enamel of unerupted molar teeth of 4–9-month-old calf embryos or 1–2-month-old postnatal calves was carefully scraped from the surface of the teeth and decalcified at 4°C in 0.5 M EDTA, pH 8.3, containing 20 mM benzamidine hydrochloride, 1 mM ethylmaleimide, 100 mM 6-aminohexanoic acid and 1 mM levamisol (Sigma, St. Louis, MO). The levamisol was added to help prevent the possible enzymatic

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dephosphorylation of the enamel phosphoproteins. After the EDTA suspension was centrifuged at 25000 rpm for 1 h, the EDTA-insoluble pellet was extracted with 0.05 M NH_4HCO_3 and the same inhibitors, dialyzed against NH_4HCO_3, centrifuged again as described above, and the solution freeze-dried. Over 95% of the protein content of the whole enamel was extracted in NH_4HCO_3 [3].

2.2. *Purification and isolation of the major, highest relative molecular mass amelogenin*

2.2.1. Gel filtration on Sephadex G-100

The NH_4HCO_3-soluble protein components of the enamel were molecularly sieved by gel filtration on a G-100 column of Sephadex (5 × 100 cm) equilibrated with 0.05 M NH_4HCO_3 and 6 M urea of 6 M Gdn-HCl (pH 8.2) at 4°C. The fractions were desalted, hydrolyzed against water and freeze-dried.

2.2.2. Ion-exchange chromatography

Fractions isolated from the G-100 column were further purified by ion-exchange chromatography on a Cellex-T column (2.5 × 12 cm) equilibrated in 0.02 M glycine NaOH, 6 M urea buffer (pH 9.2). The elution was carried out using a linear gradient of NaCl from 0 to 0.08 M over a total volume of 1200 ml and at a constant flow rate of 85 ml/h. The eluate was monitored at 280 nM. The fractions were desalted by dialysis against water and freeze-dried. The procedure was repeated two or three times until homogeneity was obtained.

2.2.3. High performance liquid chromatography

Further purification of the fractions obtained by Cellex-T ion-exchange chromatography was carried out by HPLC gel filtration on two Beckman TSK 3000 columns (0.75 × 30 cm) (Beckman Instruments, Palo Alto, CA) connected in series and equilibrated with 20 mM 2-(*N*-morpholino)ethanesulfonic acid, 2.5 mM dithiothreitol and 6 M Gdn-HCl (pH 6.5).

2.3. *Relative molecular mass determination*

2.3.1. SDS-PAGE

SDS-PAGE of purified components from Cellex-T ion-exchange chromatography and HPLC molecular sieving was carried out as described by Furthmeyr and Timpl [9] using both standard proteins and the CNBr peptides isolated and purified from the α_1 chains of rat tail tendon collagen [10,11]. The relative molecular masses were calculated by the method of Weber and Osborn [12].

2.3.2. Molecular sieving

2.3.2.1. G-100 Sephadex

The relative molecular masses of the purified components were determined by sieving through a 5 × 100 cm column of G-100 Sephadex in 0.05 M NH_4HCO_3, 6 M Gdn-HCl at pH 8.2. Standard protein components (Sigma) were used to calculate the M_r values of the enamel components.

2.3.2.2. HPLC

Two TSK 3000 columns (Beckman Instruments, Palo Alto, CA) connected in series and run at 1 ml/min and using both standard protein components and the CNBr peptides of the α_1 chain of rat tail tendon collagen [10,11] as markers were used to calculate the M_r values of the enamel components. The columns were equilibrated with 6 M Gdn-HCl (pH 6.5) containing 20 mM 2-(morpholino)ethanesulfonic acid (Sigma). The flow rate was maintained at 1 ml/min and the eluate monitored at 254 nM [13].

2.3.3. Relative molecular mass by sedimentation-equilibrium ultracentrifugation

Sedimentation-equilibrium ultracentrifugation experiments were carried out in 6 M Gdn-HCl (pH 7.0) as detailed in the appendix.

3. RESULTS

3.1. *Isolation of highest relative molecular mass, major amelogenin*

The sequential procedures of molecular sieving and repeated ion-exchange chromatography supplemented by HPLC molecular sieving resulted in the isolation of a homogeneous preparation of the highest relative molecular mass component in developing bovine enamel which had the characteristic amino acid composition of an amelogenin [3–5,7,8] (table 1). Repeated HPLC molecular sieving resulted in the elution of a single symmetrical peak. SDS-PAGE of this peak revealed a single band on SDS-PAGE. *O*-Phosphoserine (2–4 residues/1000) was identified in this major, highest relative molecular mass amelogenin.

Table 1

Major amino acids in highest relative molecular mass amelogenin isolated (residues/1000 total amino acids)

Glu	195
Pro	310
Leu	95
His	82

3.2. *Relative molecular mass*

3.2.1. SDS-PAGE

The relative molecular mass of the major, highest relative molecular mass amelogenin determined by SDS-PAGE using standard proteins as markers was approx. 25000–26000 (fig.1a). When the same component was examined in SDS-PAGE using the proline-rich CNBr peptides of the α_1 chains of rat tail tendon collagen as markers, the relative molecular mass was calculated to be 15000–17000 (fig.1b).

3.2.2. G-100 Sephadex molecular sieving

The relative molecular mass of the major, highest relative molecular mass amelogenin was calculated to be 22000–23000 when sieved in 6 M Gdn-HCl using standard proteins as markers (fig.2). CNBr peptides of α_1 chains of rat tail tendon collagen were not used as markers.

3.2.3. Relative molecular mass by sedimentation-equilibrium ultracentrifugation

The M_r of the major, highest relative molecular mass amelogenin fraction in 6 M Gdn-HCl as determined from the average of the slopes, d ln A/dr^2 = 0.53 and a partial specific volume of 0.741 is 17385.

3.2.4. HPLC molecular sieving

The M_r of the major, highest relative molecular mass amelogenin was calculated to be 26000–27000 when using standard proteins as

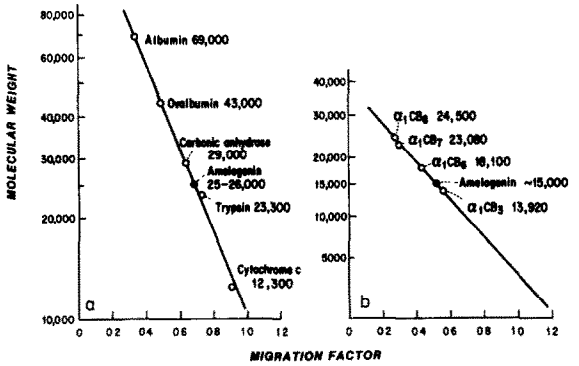

Fig.1. Relative molecular mass determination of major amelogenin by SDS-PAGE using (a) standard globular proteins as markers; (b) using CNBr peptides of α_1 chains of RTT collagen as markers.

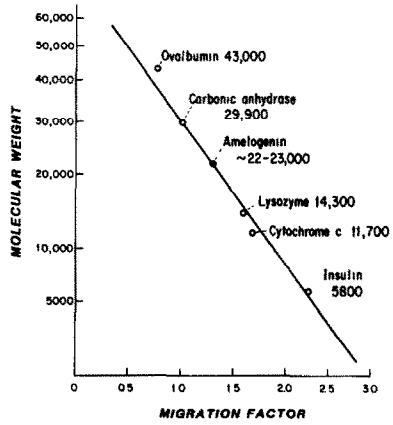

Fig.2. Relative molecular mass determination of major amelogenin by molecular sieving through a 5 × 100 cm column of G-100 Sephadex in 6 M Gdn-HCl, pH 8.2, using standard proteins as markers.

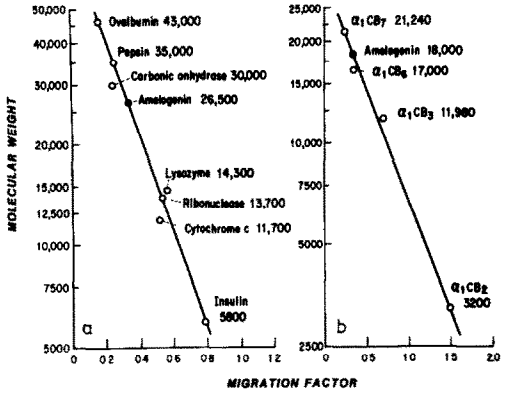

Fig.3. Relative molecular mass determination of major amelogenin by molecular sieving through two TSK 3000 columns in 6 M Gdn-HCl connected in series using (a) standard globular proteins as markers; (b) using CNBr peptides of α_1 chains of RTT collagen as markers.

markers (fig.3a) and 17000–18000 (fig.3b) when the CNBr peptides of α_1 chains of collagen were used as markers.

4. DISCUSSION

Calculation of the relative molecular masses of phosphoproteins from SDS-PAGE and molecular sieve data have given anomalous results when compared with values obtained by sedimentation equilibrium ultracentrifugation [14–16]. Here, the major and highest relative molecular mass proline-rich amelogenin component which we observed and isolated, also displayed anomalous behavior as far as calculation of its M_r is concerned using customary globular proteins as standards. The ~40% lower value for the M_r, calculated using CNBr peptides of collagen as standards, is very close to the values obtained for 4 of the major homogeneous fractions of developing bovine enamel (16100–16800) by sedimentation-equilibrium ultracentrifugation reported earlier [5], and to the value obtained in the present study by sedimentation-equilibrium ultracentrifugation. These experimental data, together with the value for the M_r calculated from recent amino acid sequence studies (~19000) [17], lead us to conclude that the true M_r of this major, high M_r amelogenin, falls in the range of 15000–19000. Thus, as far as the major question posed, namely, what is the relationship between the previously described 15000–17000-Da amelogenin components and the more recently isolated component of allegedly 26000–30000-Da which was presumed to be a new high M_r amelogenin, we conclude that they are the same component(s), the recently reported M_r of 26000–30000 [7,8] being in error. Moreover, our unpublished results confirm the earlier work of Eggert et al. [5] and the more recent work of Fincham et al. [8] that this component is not homogeneous but consists of a number of closely related species with similar amino acid compositions. No data are available at this time as to how many of these closely related proteins derive from a single or several separate gene products.

APPENDIX

A model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA), equipped with a xenon light source, a monochromator, a mirror optical system, and a photoelectric scanner, was used. The signal which operated the scanner recorder pen was amplified and supplied to the A/D circuit of a PDP 11/03 computer, where the signal was digitized and stored on a flexible disk. The data were analyzed using a VAX 780 computer. Protein concentrations of 1.6 and 1.1 mg/ml were used in the two sets of experiments that were performed. A solution volume of 0.1 ml and a rotor speed of 30000 rpm were used. The attainment of sedimentation-equilibrium was found to take place in 40 h, beyond which time no changes in sedimentation patterns were observed. Relative molecular masses were determined from the slopes of the plots of ln A vs r^2 as follows:

$$M = \frac{\mathrm{d} \ln A}{\mathrm{d}r^2} \frac{2RT}{\omega^2} / (1 - \phi' \varrho)$$

where M is the M_r, A and r are absorbance and radial distance, respectively, R is the gas constant, T is the absolute temperature, ω is the rotor speed; ϕ' is the apparent partial specific volume of the protein, and ϱ is the density of the solution and was assumed to be 1.143.

Calculation of partial specific volume of amelogenin

The partial specific volume, $\bar{\nu}$, of bovine amelogenin [17] was calculated using the method of Cohn and Edsall [18]:

$$\bar{\nu}_i = \frac{\sum_i N_i (W_i \bar{\nu}_i)}{\sum_i N_i W_i}$$

where N_i = number of residues of amino acid of type i; W_i = residue relative molecular mass of the amino acid of type i minus 1 mol of water; $\bar{\nu}_i$ = partial specific volume of the amino acid of type i. In the calculation, the values of N_i from [17] were used. The values for $\bar{\nu}_i$ of the amino acid residues were adapted from [19] except for $\bar{\nu}_i$ 0.50 of Ser(P) [20]. These calculations gave a value of $\bar{\nu} = 0.74$, essentially identical to the value calculated from the amino acid composition of the samples used for the ultracentrifugation studies. The apparent partial specific volume, ϕ', is related to the partial specific volume $\bar{\nu}_i$, by:

$$\phi' = \bar{\nu} - (1/\varrho - \bar{\nu}_3)(A_3 - g_3 A_1)$$

where ϱ and $\bar{\nu}_3$ for 6 M Gdn-HCl are 1.1418 g/ml at 20°C and 0.763 ml/g, respectively. The parameter, g_3, is defined by solvent composition; for 6 M Gdn-HCl, its value is 1.007 g Gdn-HCl per g water [21]. The parameter, A_1, is related to the hydration of the protein and is computed from the hydration of the constituent amino acid residues [22]. The parameter, A_3, accounts for the extent of Gdn-HCl binding to protein in 6 M Gdn-HCl and is calculated empirically [21]. The value of ϕ' is calculated to be 0.741.

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REFERENCES

[1] Eastoe, J.E. (1960) Nature 187, 411–412.
[2] Glimcher, M.J., Mechanic, G., Bonar, L.C. and Daniel, E.J. (1961) J. Biol. Chem. 236, 3210–3213.
[3] Glimcher, M.J., Mechanic, G.L. and Friberg, U.A. (1964) Biochem. J. 93, 198–202.
[4] Robinson, C., Lowe, N.R. and Weatherell, J.A. (1977) Calcif. Tiss. Res. 23, 19–31.
[5] Eggert, F.M., Allen, G.A. and Burgess, R.C. (1973) Biochem. J. 131, 471–484.
[6] Seyer, J.M. and Glimcher, M.J. (1977) Calcif. Tiss. Res. 24, 253–257.
[7] Termine, J.D., Belcourt, A.B., Christner, P.J., Conn, K.M. and Nylen, M.U. (1980) J. Biol. Chem. 255, 9760–9768.
[8] Fincham, A.G., Belcourt, A.B., Termine, J.D., Butler, W.T. and Cothran, W.C. (1983) Biochem. J. 211, 149–154.
[9] Furthmeyr, H. and Timpl, R. (1971) Anal. Biochem. 41, 510–516.
[10] Butler, W.T., Piez, K.A. and Bornstein, P. (1967) Biochemistry 6, 3771–3779.
[11] Bornstein, P. (1969) Biochemistry 8, 63–71.
[12] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[13] Ui, N. (1979) Anal. Biochem. 97, 65–71.
[14] Stetler-Stevenson, W.G. and Veis, A. (1983) Biochemistry 22, 4326–4335.
[15] Jontell, M., Pertoft, H. and Linde, A. (1982) Biochim. Biophys. Acta 705, 315–320.
[16] Creamer, L.K. and Richardson, T. (1984) Arch. Biochem. Biophys. 234, 476–486.
[17] Takagi, T., Suzuki, M., Baba, T., Minegishi, K. and Sasaki, S. (1984) Biochem. Biophys. Res. Commun. 121, 592–597.
[18] Cohn, E.J. and Edsall, J.T. (1965) in: Proteins, Amino Acids, and Peptides, pp.370–381, Reinhold, New York.
[19] Lee, J.C. and Timasheff, S.N. (1979) Methods Enzymol. 61, 49–57.
[20] McMeekin, T.L., Groves, M.L. and Hipp, N.J.J. (1949) J. Am. Chem. Soc. 71, 3298–3300.
[21] Lee, J.C. and Timasheff, S.N. (1974) Biochem. 13, 257–265.
[22] Kuntz, J.D. (1971) J. Am. Chem. Soc. 93, 514–516.