Phosphohydrolase Activities in Developing and Mature Dental Tissues

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In the course of the odontogenesis of bovine incisors several clearly distinguishable phosphohydrolase activities are observed in the pulp and in dental hard tissues. Using various substrates and inhibitors, unspecific alkaline phosphatase, two isoenzymes of acid phosphatase, Ca^{2+}-activated ATPase and inorganic pyrophosphatase are characterized. The enzymatic activity of alkaline phosphatase in pulp and hard tissues is significantly high at the beginning of dentine and enamel mineralization. The specific activity of this enzyme decreases quite fast with the beginning of root formation, then more slowly, until it reaches a constant final value. Histochemical studies show that during mineralization the maximum of alkaline phosphatase activity is in the subodontoblasts. Lower enzyme concentrations are found in the stratum intermedium and in the outer enamel epithelium during that process.

The specific activities of ATPase, acid phosphatases and pyrophosphatase show little temporal variation during tooth development, but they also appear in a characteristic spatial pattern in the dental tissues.

Introduction

The mineralization which causes the formation of biological hard tissues is not a simple precipitation from an oversaturated solution. It rather proceeds on an extracellular organic network which consists of phospho- and glycoproteins of unusual composition [1–7]. The role of these matrix proteins during hard tissue formation, especially during mineralization, is still obscure.

Two functional sets of enzymes are of primordial importance for odontogenesis. The first one is correlated with the formation and decomposition of the protein matrix, such as proteinkinases, glycosyltransferases and proteases. The second group affects the calcium and phosphate metabolism and is involved in the formation of hydroxylapatite, the main mineral of dental hard tissues.

Phosphatases [1–18], ATPases [19–23] and pyrophosphatase [24, 25] are found in different soft and hard tissues of growing and mature teeth and first results show an increase of phosphatase production during certain stages of odontogenesis. We have now studied in detail the temporal changes of phosphohydrolases in the course of tooth development of one single mammal. Incisors of calves and young cattle were used, since the development of bovine incisors resembles the human odontogenesis: Both species represent diphyodonts and exhibit dental matrix proteins of similar size and amino acid composition [26–29]. Soft and hard tissues of every developmental stage from toothgerm to completely mineralized teeth were studied enzymatically and histochemically. In this paper we report about the occurrence, localization and properties of several phosphate releasing enzyme activities during odontogenesis.

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Materials and Methods

Toothgerms and partially or completely mineralized incisors were removed from the jaws of 18 to 36 month old cattle. Gum tissue was removed carefully and the pulp with adhering odontoblasts was detached from the hard tissue. The separated material was stored at −78 °C unless it was used immediately.

Sample preparation of pulpal tissue

Enzyme activities in the pulp were measured in freshly prepared crude extracts. For that purpose pulp tissue was weighed and homogenized in 3 to 20 volumes of distilled water with a Dounce homogenizer or an Ultraturrax at 0 °C. After 30 min of incubation at 25 °C collagen fibers and large organelles were removed by centrifugation at 15,000 × *g* at 0 °C. The supernatant was stored at 0 °C during experiments.

Specific activities are based on protein concentrations determined by the method of Bradford [30].

Sample preparation of mineralized tissue

Deep frozen dental hard tissues were pulverized by means of a rotary beater mill (Pulverisette 14, Fritsch GmbH, Idar-Oberstein) while cooling with dry ice. The cylindrical sieves and the rotor were cooled beforehand with dry ice to minimize the inactivation of enzymes in the dental tissue. By the use of sieves with 80 µm, 200 µm, 500 µm, 1 mm and 4 mm nominal pore width material with different grain size distribution was obtained. The powder was dried over silica gel in a desiccator at 4 °C and stored at −78 °C.

For enzyme assays samples of powdered hard tissue were homogeneously suspended in test buffer using an electrically driven stirring blade in the fluorometer or a magnetic stirrer (Hellma cuv-O-stir model 333) in the case of absorbance measurements. The enzymatic test was started by addition of substrate containing solution. For blank values pulverized dental hard tissue was heated to 140 °C for 10 min.

Assays for alkaline phosphatase

p-Nitrophenylphosphate hydrolysis was measured in 0.5 M diethanolamine/HCl buffer pH 9.0, containing 0.5 mM $MgCl_2$, following the increase in absorbance at 405 nm at 25 °C (ε 18,800 $M^{-1} \cdot cm^{-1}$) [31, 32]. In the blanks heat denatured protein was used or levamisole was added to a final concentration of 3 mM. For the concentration of different effectors used in the study of alkaline phosphatase see Table II.

Tests with 4-methylumbelliferylphosphate as substrate were carried out on a Perkin Elmer fluorescence spectrophotometer MPF 4 or a Beckman fluorometer 1076. Excitation and emission wavelengths were 362 nm and 445 nm, respectively [33, 34]. To estimate the activity per volume unit and the specific activity standard curves of the product 4-methylumbelliferone were generated in the concentration range from 5 nM to 25 µM. In absorbance measurements of 4-methylumbelliferylphosphate hydrolysis a molar extinction coefficient of 17,300 at 360 nm was used.

In assays of alkaline phosphatase activity with pyrophosphate or other non-chromogenic or nonfluorogenic substrates the amount of released *ortho*-phosphate was determined according to Granström [9]. A calibration curve was obtained with 0−0.1 mM KH_2PO_4. For the blank values 20% trichloroacetic acid was added prior to incubation with enzyme.

Coupled assays for phosphatase activity in mineralized dental tissues with different substrates

The assay mix was stirred at room temperature in a cuvette: 5 mg pulverized hard tissue (particle size ≧80 mesh) was suspended in 2.0 ml of 0.5 M diethanolamine/HCl pH 9.0, 0.5 mM $MgCl_2$ and 3 mM NAD^+; 0.1 ml enzyme solution was added first, then 100 µl 0.1 M substrate solution in buffer pH 9.0. The reaction was followed at 366 nm in a photometer "Eppendorf". We used:

A) glucose-6-phosphate, 1-α-phosphoglucose and 1-β-phosphoglucose with glucose dehydrogenase from *Bacillus megaterium* (90 U/ml),
B) L-α-glycerol-3-phosphate with glycerol dehydrogenase from *Enterobacter aerogenes* (250 U/ml),
C) ethylphosphate with alcohol dehydrogenase from yeast (400 U/ml) or
D) *n*-butylphosphate with alcohol dehydrogenase from horse liver (45 U/ml).

Assays for inorganic pyrophosphatase

The velocity of pyrophosphate hydrolysis was determined by measuring the amount of inorganic phosphate released [9] or by spectrofluorometry using a modified assay of Cooperman *et al.* [35]: 3 mM inorganic pyrophosphate pH 8.0 in presence of 2 mM Mg^{2+} ions and 1 µM 4-methylumbelliferone as pH-dependent fluorescence indicator. A calibration curve was obtained from a gradient generated from the magnesium pyrophosphate solution described above and a sample of this solution which had been completely hydrolyzed. Hydrolysis was performed by the aid of inorganic pyrophosphatase until no further decrease in pH and fluorescence was observable, thereafter the solution was heated to 100 °C for 5 min in order to destroy any enzyme activity. The calibration curve correlating pH- and fluorescence-decrease with pyrophosphate hydrolysis is almost linear downward to pH 7.6, a value indicating the release of 0.6 mM of inorganic phosphate. Using a concentration of 100 µM 4-methylumbelliferone the system could be used for spectrophotometric meas-

urements of pyrophosphatase activity at 362 or 366 nm.

The concentration of phosphatase inhibitors used to distinguish inorganic pyrophosphatase from other phosphohydrolases are included in Table VIII.

Assays for acid phosphatase

The activity was measured with *p*-nitrophenylphosphate as substrate in 0.1 M acetate buffer pH 4.2 [36]. When non-chromogenic substrates were used, the activity was determined on the basis of inorganic phosphate released as described previously.

Assays for ATPase

ATPase activity was determined either according to the bioluminescence method [37, 38] or by measuring the amount of released inorganic phosphate [39].

Histochemical localization of phosphohydrolase activities

For histochemical analysis the dental tissue was embedded in bovine liver, frozen in liquid nitrogen, stored at −78 °C and cut into 10−40 µm slices on a microtome. Samples of dental tissue were prefixed in 2.5% glutaraldehyde for 18 h for controls. Tissue slices were tested for background of inorganic phosphate. Other control slices were preincubated in 15% acetic acid for 10 min.

A modified method of Gomori [40] was used for the staining of alkaline phosphatase activity with β-glycerophosphate as substrate [41]. Different phosphohydrolase activities were distinguished by addition of several inhibitors to the incubation mix: levamisole (3 mM), cysteine (3 mM), ouabain (1 mM), fluoride (10 mM) or tartrate (10 mM).

Staining for acid phosphatases was done by simultaneous azo coupling according to Barka and Anderson [42], ATPases were stained in an incubation medium described by Lojda *et al.* [43].

Results

1. Isolation, analysis, characterization and differentiation of phosphohydrolases

All phosphohydrolase activities of bovine pulp were measured in aqueous extracts using standard conditions. Thus the results of different measurements are comparable. Characterization and differentiation of single enzyme/isoenzyme activities was attempted by the addition of various substances, known to inhibit individual enzymes. A comparable procedure for the quantitation of phosphohydrolases in dental hard tissues is given in the following paragraph.

1.1 Alkaline phosphatase

1.1.1 Calibration experiments

Alkaline phosphatase activity in dental hard tissues were measured in suspensions of finely grinded dentine and enamel. *p*-Nitrophenylphosphate or 4-methylumbelliferylphosphate were substrates in photometric assays. The latter fluorogenic substrate was even suitable to detect trace amounts of phosphatase activity. As shown in Fig. 1 the time

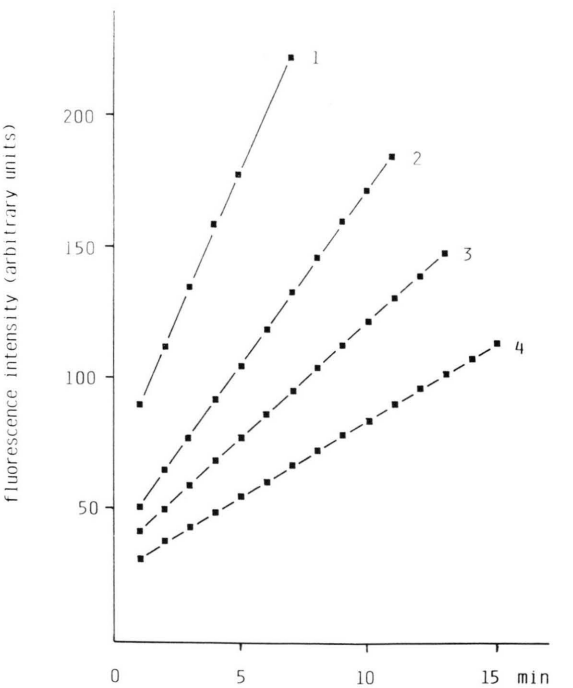

Fig. 1. Initial progress curve of 4-methylumbelliferylphosphate hydrolysis catalyzed by alkaline phosphatase activity of a powdered dentine/enamel sample. The weight of suspended dental hard tissue in 2 ml of the stirred test buffer pH 9.0 was: 1 = 10.1 mg; 2 = 6.1 mg; 3 = 4.2 mg; 4 = 2.6 mg. The reaction was started by addition of the fluorogenic substrate; the fluorescence of 4-methylumbelliferone was measured at 445 nm using an excitation wavelength of 362 nm. Ordinate: Fluorescence intensity at 445 nm; abscissa: reaction time.

curves of hydrolysis show a strictly linear initial part and the resulting reaction velocities are proportional to the absolute amount of suspended dental tissue (Fig. 2). The phosphatase activity was determined quantitatively, if the particle size of the dental powder was ≦0.5 mm (Fig. 3). Thus the diffusion of substrate or product between the surface of minute particles and enzymes included therein is no longer rate limiting. During experiments the phosphatase remains almost completely bound to the suspended hard tissue, only a small part of enzyme is solubilized: By solubilization with *n*-butanol only 5 to 10% of alkaline phosphatase is isolated from the dental hard tissue.

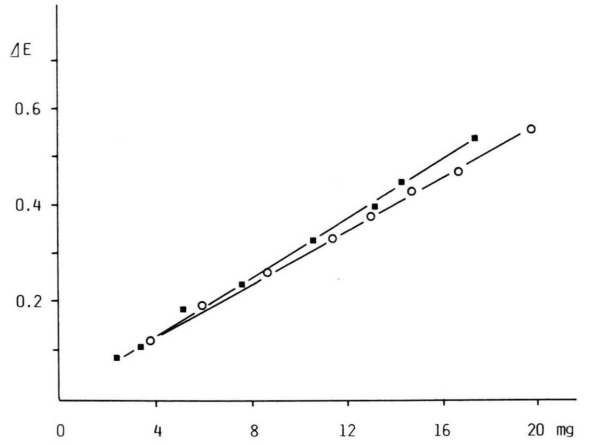

Fig. 2. Comparative hydrolysis of *p*-nitrophenylphosphate (■) and 4-methylumbelliferylphosphate (○) catalyzed by alkaline phosphatase activity of a dentine/enamel sample at pH 9.0; ordinate: absorbance increase/15 min at 405 nm (■, ε = 18,300) and 360 nm (○, ε = 17,300) respectively; abscissa: weight of the suspended dentine/enamel sample in mg.

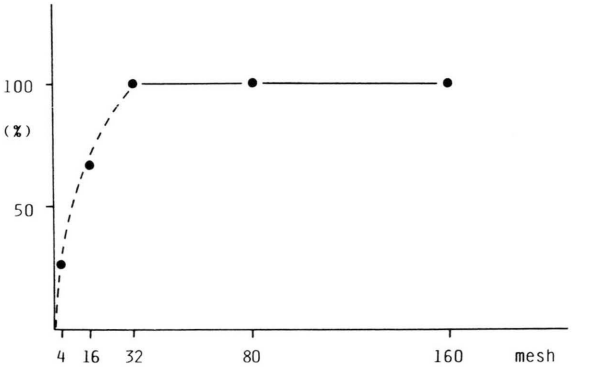

Fig. 3. Alkaline phosphatase activity of dentine/enamel samples *versus* nominal particle size. Test substrate: 4-methylumbelliferylphosphate dissolved in diethanolamine/HCl buffer pH 9.0; the activity is derived from the fluorescence increase at 445 nm (excitation wavelength 362 nm). Ordinate, relative activity; abscissa: reciprocal values of the particle size in mesh.

Table I. Alkaline phosphatase activity of dentine/enamel samples measured with 4-methylumbelliferylphosphate in presence or absence of surface active compounds.

Detergent	Relative activity [%]
Control (no additive)	100
1 % *n*-Octyl-β-D-glucoside	98
1 % 1-Butanol	92
1 % Polyoxyethylenesorbitan monolaurate	90
1 % *n*-Octyl-β-D-thioglucoside	80
1 % Sodium dodecyl sulfate	78

Addition of detergents did not increase the enzyme activity of suspended tissue samples (Table I). Therefore no surface active compounds were used in further measurements.

1.1.2 *In vitro* studies of alkaline phosphatase

Initial studies of alkaline phosphatase were performed with artificial substrates, *p*-nitrophenylphosphate and 4-methylumbelliferylphosphate. The pH optimum of alkaline phosphatase from pulp lies between pH 9 and 10, depending on the substrate. The activity found in pulp extracts is affected by detergents. Addition of β-octylglucoside or Triton X-100 increases the activity of pulp extracts by 5 to 20% (Fig. 4), but the enzyme is less stable. The activity decreases by 30% within 2 days in contrast to a loss of only 5% per month (at 4 °C) in the control without surfactant. Since more than 80% of the pulp enzyme could be solubilized without addition of detergent, all following experiments were conducted in the absence of such compounds.

The influence of different effectors on the activity of alkaline phosphatase of bovine pulps was studied with *p*-nitrophenylphosphate as substrate. The results listed in Table II show that the sensitivity of the isozyme from pulp is not the same as that of alkaline phosphatases from other tissues. Characteristic is the strong inhibition by low concentrations of levamisole [44, 45], indicating that other

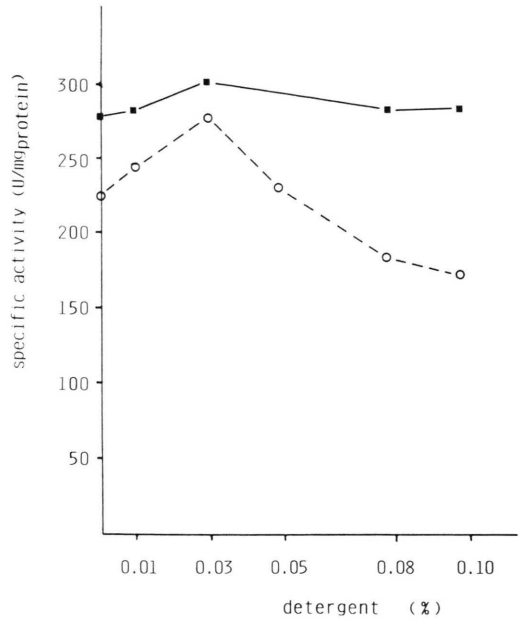

Fig. 4. Influence of detergents on the specific activity of alkaline phosphatase in extracts from the pulp of calf incisors. Test substrate: *p*-nitrophenylphosphate in diethanolamine/HCl buffer pH 9.0; O– – –O Triton X-100; ■——■ β-octylglucoside.

Table II. Influence of several effectors on the activity of alkaline phosphatase from pulp (*p*-nitrophenylphosphate as substrate).

Effector	Final concentration [mM]	Relative activity [%]
–	–	100
Mg^{2+}	1.0	137
Zn^{2+}	0.5	9
Levamisole	0.01	50
	3.0	0
EDTA	100.0	1
L-Phenylalanine	1.0	100
	2.0	100
Fluoride	0.1	101
Molybdate	10.0	19
Phosphate	10.0	30
Vanadate	0.05	54
	1.0	4
L-(+)-Tartrate	10.0	103
Cyanide	2.0	56
Imidazole	16.0	16

phosphohydrolytic activities on *p*-nitrophenylphosphate can be neglected at alkaline pH: 50% inhibition is achieved by 10 μM levamisole. Inhibition by molybdate and phosphate is significantly less pronounced and requires millimolar concentrations of these compounds. Fluoride and tartrate ions do not affect the enzyme activity. Alkaline phosphatase from bovine pulp hydrolyzes a series of phosphoric acid esters and anhydrides (Table III). Addition of levamisole to the assay mix allowed the differentiation of alkaline phosphatase from other phosphohydrolases. Especially high is the activity of the pulp enzyme with *p*-nitrophenylphosphate and 4-methylumbelliferylphosphate. Both substrates are hardly converted in the presence of levamisole. The total activity of alkaline phosphatase is represented by the levamisole inhibitable activity on *p*-nitrophenylphosphate or 4-methylumbelliferylphosphate. Other substrates shown in Table III are notably hydrolyzed even in the presence of 3 mM levamisole. The results indicate that compounds such as ATP, glycerophosphate and inorganic pyrophosphate are hydrolyzed to a considerable extent by pulp enzymes different from alkaline phosphatase. Glucose-6-phosphate is not converted by alkaline phosphatase from bovine pulp to a measureable degree.

Resembling the enzyme from pulp, the non-physiological substrates *p*-nitrophenylphosphate and 4-methylumbelliferylphosphate are also rapidly hydrolyzed by alkaline phosphatase from dental hard tissue (dentine or enamel). The physiological substrate L-α-glycerophosphate was hydrolyzed more slowly as compared to the aromatic esters mentioned above; data found with a single dentine sample are shown in Table IV.

Table III. Substrate specificity of alkaline phosphatase from pulp.

Substrate	Relative activity [%] without inhibitor	with 3 mM levamisole*
p-Nitrophenylphosphate	100	1
4-Methylumbelliferylphosphate	86	0
ATP	46	26
Inorganic pyrophosphate	20	29
β-Glycerophosphate	46	26
Glucose-6-phosphate		
Phosphothreonine		
Phosphotyrosine		
Phosphoserine		

n.d.: not determined; * values in this lane are given as percentage of the relative activity of alkaline phosphatase with the same substrate in absence of inhibitor.

Table IV. Substrate specificity of alkaline phosphatase from bovine dentine*.

Substrate	Relative activity [%]
p-Nitrophenylphosphate	100
4-Methylumbelliferylphosphate	95
L-α-Glycerol-3-phosphate	55
α-D-Glucose-1-phosphate	0
β-D-Glucose-1-phosphate	0
Glucose-6-phosphate	0
Ethylphosphate	0
n-Butyl-phosphate	0
Inorganic pyrophosphate	<1**

* The sample was prepared from permanent incisors in the stage of crown mineralization; ** lit. cit. [46].

No phosphohydrolase activity at all was observed with dental hard tissue and α-D-glucose-1-phosphate, β-D-glucose-1-phosphate, glucose-6-phosphate, ethylphosphate and *n*-butylphosphate (Table IV).

No pyrophosphatase hydrolysis attributable to alkaline phosphatase was detected in suspensions of dentine and enamel by standard assay techniques such as pH-stat measurements or with sensitive pH-dependent fluorophores. Recent studies using HPLC techniques indicate the upper limit of pyrophosphatase activity given in Table IV [46].

1.2 Adenosintriphosphatase

Small amounts of ATPase are found in the pulp as well as in hard tissues of bovine incisors. The highest specific activity observed in extracts from pulp was 10 mUnits/mg; only 0.2 mU/mg are found in hard tissue. The Ca^{2+}-ATPase is not inhibited by ouabain and is thereby distinguished from the Na^+/K^+-ATPase. The distinction between ATPase and phosphatase activity is accomplished by applying the inhibitors levamisole, tungstate and molybdate, which merely affect phosphatase activity (Table V).

Table V. Inhibition of total ATPase activity from pulp.

Inhibitor	Concentration [mM]	Relative activity [%]
Without inhibitor	–	100
Molybdate	0.10	89
Tungstate	0.10	80
Levamisole	0.10	88
Ouabain	0.50	99

1.3 Acid phosphatase

Acid phosphatase from bovine pulp hydrolyzes *p*-nitrophenylphosphate with a pH-optimum of 4.9. The enzyme is inhibited by molybdate, fluoride and tartrate (Table VI).

Since levamisole does not inhibit acid phosphatase, this compound was added to the assay mix in order to suppress alkaline phosphatase activity completely. The activity of acid phosphatase from bovine pulp with *p*-nitrophenylphosphate as substrate is high compared to the activity of the enzyme in other tissues. A specific activity ranging from 2 to 7 mU/mg protein represents approximately 10% of the activity found in the prostate gland. The substrate specificity of the enzyme is low: it hydrolyzes *p*-nitrophenylphosphate, ATP, β-glycerophosphate and several phosphorylated amino acids at about the same rate (Table VII). The activity of acid phosphatase in dental hard tissue is very low. Only 0.03 mU/mg protein were

Table VI. Inhibition of the acid phosphatase activity from the pulp of bovine incisors.

Inhibitor	Concentration [mM]	Relative activity [%]
Without inhibitor	–	100
Fluoride	0.5	44
L-Tartrate	8.0	69
	10.0	60
Molybdate	0.0004	46
Inorganic phosphate	5.0	85
	20.0	77
	40.0	72
Levamisole	3.0	97
L-Cysteine	0.4	96
Ouabain	0.03	77
Mg^{2+}	1.0	100

Table VII. Relative activity of acid phosphatase from bovine pulp with different substrates.

Substrate	Concentration [mM]	Relative activity [%]
p-Nitrophenylphosphate	3.0	100
ATP	1.0	88
β-Glycerophosphate	1.0	88
O-Phosphothreonine	1.0	75
O-Phosphoserine	1.0	88
O-Phosphotyrosine	1.0	88
Inorganic pyrophosphate	1.0	50

found at pH 4.2 in the presence of 3 mM levamisole with *p*-nitrophenylphosphate as substrate.

1.4 Inorganic pyrophosphatase

Specific inorganic pyrophosphatase is distinguished from acid and alkaline phosphatase by the use of different phosphatase inhibitors. The "*p*-nitrophenylphosphatase activity" was studied in parallel to obtain data concerning the inhibition of phosphatases. Table VIII shows the influence of different effectors on the rate of hydrolysis of inorganic pyrophosphate and *p*-nitrophenylphosphate in the presence of pulp extracts. The results show, that inorganic pyrophosphate is hydrolyzed by a specific inorganic pyrophosphatase and, to a lesser extent by alkaline phosphatase. The specific activity of inorganic pyrophosphatase in bovine pulp is very low. Values varying from 0.4 to 0.8 mU/mg protein are found in permanent and deciduous incisors.

2. Histochemical analysis and enzyme distribution during tooth development

2.1 Temporal variation of phosphohydrolase activities

2.1.1 Pulpal enzymes

If phosphohydrolases play a special role in the mineralization process one expects quantitative changes of the respective specific and total activities during odontogenesis. Therefore we studied the pulps of toothgerms at different developmental stages. The age of a tooth was expressed as ratio of the length of the root and the total length. In Fig. 5 is shown that the highest activity of alkaline phosphatase occurs prior to root formation during the mineralization of the crown of the tooth. The specific activity diminishes rapidly in the course of root formation to a level typical for pulps of mature teeth.

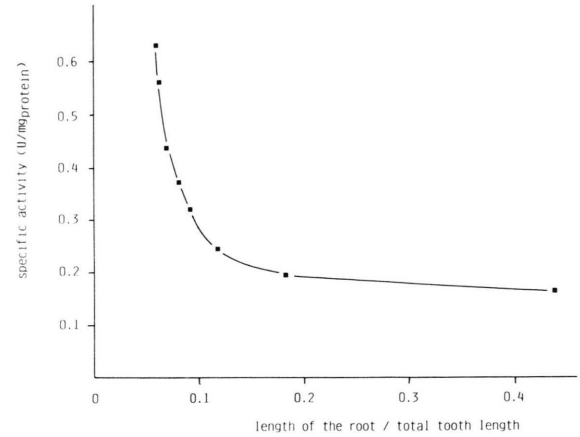

Fig. 5. Dependency of the specific activity of alkaline phosphatase from pulp on the developmental stage of the tooth. Test substrate: *p*-nitrophenylphosphate in diethanolamine/HCl buffer pH 9.0.

As compared to alkaline phosphatase the time-defined variation of Ca^{2+}-ATPase from bovine pulp is considerably less pronounced. The activity in pulp extracts drops from 10 milliunits/mg protein in developing teeth to 3 mU/mg in fully mineralized mature teeth.

Table VIII. Influence of inhibitors on pyrophosphatase and *p*-nitrophenylphosphatase activity in bovine pulp.

Inhibitor	Concentration [mM]	Relative activity against PP [%]*	*p*NPP [%]**
Without inhibitor	–	100	100
Fluoride	0.1	95	101
Molybdate	10.0	0	19
meso-Tartrate	10.0	94	103
Cyanide	2.0	70	56
Imidazole	16.0	84	16
Levamisole	3.0	75	0
Glycine	100.0	91	n.d.
Mg^{2+}	3.0	100	410
Ascorbic acid	5.0	190	n.d.

* PP = inorganic pyrophosphate; ** *p*NPP = *p*-nitrophenylphosphate; n.d. = not determined.

No clear-cut temporal trend attributable to odontogenesis and aging of the teeth was observed in the case of acid phosphatase and inorganic pyrophosphatase: The enzyme activity of acid phosphatase varies from 3 to 7 mU/mg protein in deciduous teeth of 18 to 36 month old cattle. In permanent incisors the activity is nearly constant with about 2–3 mU/mg protein. Activity of inorganic pyrophosphatase is nearly constant during tooth development too. Completely developed and mineralized incisors showed slightly higher activities as compared to tooth germs or teeth in the course of mineralization.

2.1.2 Enzyme variation in dental hard tissue

In the course of odontogenesis the total activity of alkaline phosphatase changes similarly in hard tissue and pulp, if the total activity is correlated to the weight of the hard tissue. In this regard it is to be considered that the weight of a tooth increases significantly during maturation by growth and embedding of mineral. The determination of specific activities is useless for the hard tissue, because the total amount of structural proteins of the dental matrix increases in parallel to the tooth growth. The phosphatase activities in deciduous and permanent incisors were correlated with the age of the calves as summarized in Fig. 6. The highest enzyme activity was observed during mineralization of the crown of permanent incisors with a value of 8.5 mU/mg dental hard tissue. During root formation the activity decreases to 10–50% of the maximum and amounts to 1–5% of this value in fully erupted permanent incisors as well as in mature deciduous teeth.

Acid phosphatase and ATPase activities in hard tissue did not change significantly in the course of tooth development.

2.2 Local distribution of phosphohydrolase activities

2.2.1 Intracellular distribution

Initial studies on the intracellular distribution of alkaline phosphatase showed the presence of high activity of this enzyme in the pulps of developing bovine incisors and in agreement with Harada *et al.* [12] we found activity in all cellular fractions (Table IX). A large part of the total activity is located in the mitochondria, the highest specific ac-

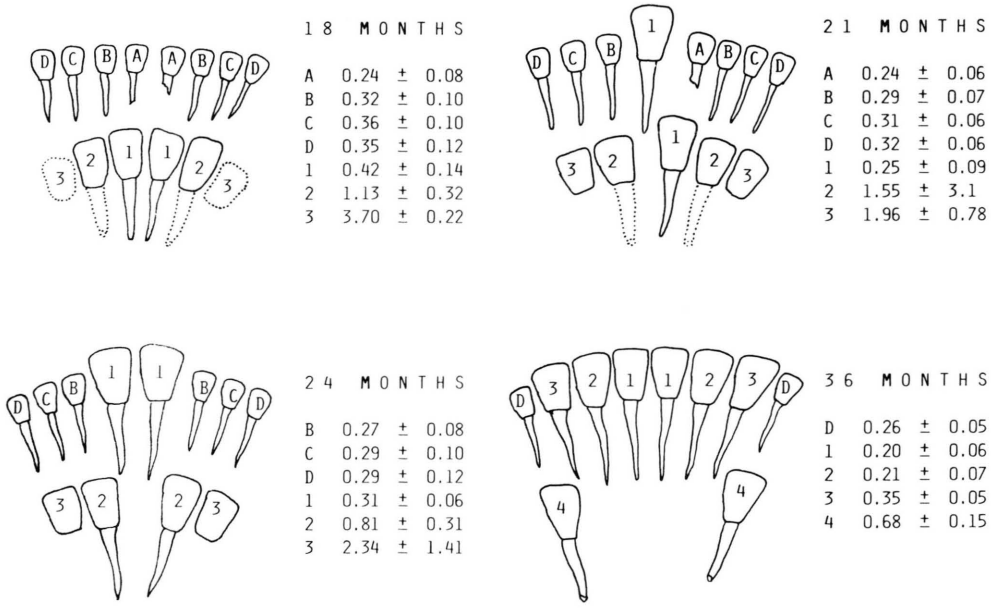

Fig. 6. Distribution of alkaline phosphatase activity in the hard tissue of bovine incisors of different age and developmental stage. Test substrate: *p*-nitrophenylphosphate in diethanolamine/HCl buffer pH 9.0. Deciduous teeth are marked with A to D, permanent incisors with 1 to 4. Listed are mean values of the activity of 8 specimen.

Table IX. Intracellular distribution of alkaline phosphatase activity from pulp.

Fraction	Total activity [units]	Total protein [mg]	Specific activity [units/$mg_{protein}$]
Homogenate	6.96	40.4	0.174
Nuclei	0.86	10.7	0.081
Mitochondria	2.18	13.8	0.160
Microsomes	0.13	0.1	1.331
Supernatant	1.03	18.4	0.057

tivity in the microsomal fraction. The alkaline phosphatase activity present in the microsomal fraction amounts to only 2% of the total activity.

2.2.2 Histochemical studies

Histochemical studies on alkaline phosphatase with β-glycerophosphate as substrate show a characteristic distribution of the enzyme activity (Table X). The absence of the enzyme in ameloblasts is a remarkable feature during all stages of tooth development. In the enamel organ the activity is predominant in the cells of the stratum intermedium, and the outer enamel epithelium shows high enzyme activity too. The pulp and its precursor have low activity during all stages. The subodontoblasts are rich in alkaline phosphatase from the beginning of matrix secretion, whereas alkaline phosphatase in the odontoblasts could not be demonstrated before mineralization. In this region the von Korff fibers, which are situated between the odontoblasts in direction to the predentine, are enzymatically very active.

The distribution of Ca^{2+}-ATPase resembles that of alkaline phosphatase. Histochemically the enzyme activity is demonstrated in odontoblasts, subodontoblasts and in the walls of the blood vessels in the pulp.

The temporal course of occurrence and localization of acid phosphatase during odontogenesis was studied histochemically in toothgerms with β-glycerophosphate as substrate. During bovine enamel formation acid phosphatase activity was first observed at the beginning of the secretion of the protein matrix (Table XI). At this stage the highest activity is found in the ameloblasts, similar to human odontogenesis [47]. In the stratum intermedium and in the odontoblasts acid phosphatase

Table X. Histochemical study of alkaline phosphatase activity in bovine tooth germs.

Cell type	Before secretion	During of the matrix	During mineralization
Outer enamel epithelium	++	++	++
Stratum reticulare	+	+	+
Stratum intermedium	+++	+++	+++
Ameloblasts	–	–	–
Odontoblasts	*	–	++
Subodontoblasts	–	+++	++++
Pulp	+	+	+
Blood vessels	++	++	++

* Not differentiated; – to ++++ increasing activity according to formed CoS; controls were incubated in presence of 3 mM levamisole.

Table XI. Histochemical study of acid phosphatase activity in the development of permanent bovine incisors.

Cell type	Before matrix secretion	During matrix secretion	During dentine and enamel mineralization	After dentine and enamel mineralization
Ameloblasts	–	++	+	+
Stratum intermedium	++	++	+	+
Odontoblasts	+	++	++	+

– Not detectable; +, ++ increasing activity.

activity is present before matrix formation begins. The activity increases during matrix secretion in the odontoblasts and is high in the stratum intermedium, too (Table XI).

Acid phosphatase activity in the pulp, ameloblasts and stratum intermedium can be completely inhibited by tartrate or fluoride. In the stratum reticulare, however, only a part of the enzyme activity is inhibited by high concentrations of these ions. The acid phosphatase in the outer enamel epithelium appears to be fluoride and tartrate insensitive.

Discussion

The matrix formation and mineralization are extracellular processes at the border between the enamel organ and the pulp. Related enzymes do not have to be locally active but can produce transportable metabolites in remote regions. Therefore, our investigation on the role of enzymes in odontogenesis covers all dental tissues. Since the pulp shows high concentrations of enzymes compared to the partially or completely mineralized hard tissues, we determined the various phosphohydrolase activities of these tissues in different experiments. The separate treatment was necessary, too, because the pulp enzymes could be solubilized almost quantitatively, whereas phosphohydrolase activities were only partially extracted from mineralized tissue. Yields rarely exceeded 10% in the latter case, a result comparable to the isolation of alkaline phosphatase from the dentine of rabbit incisors [11]. For quantitative determination of enzymes in dental hard tissues, samples were pulverized at low temperature and suspended in assay mix. The calibration experiments performed with *p*-nitrophenylphosphate at alkaline pH (Fig. 1, 2) show that alkaline phosphatase, a membrane associated enzyme, can be measured with great accuracy. The same should be valid for other phosphohydrolases, too. The biochemical tests for enzyme activities in pulp and hard tissues were complemented by histochemical studies which contributed to the knowledge of the distribution of particular enzymes.

A Alkaline phosphatase

In laboratory routine tests this enzyme activity is usually characterized by the aid of artificial substrates: *p*-nitrophenylphosphate in photometric assays or 4-methylumbelliferylphosphate in absorbance and fluorescence measurements. Our study on the substrate specificity of the enzyme from bovine pulp shows that both compounds are indeed rapidly hydrolyzed. Naturally this fact cannot give any hint as to the role of this enzyme *in vivo*. Phosphoric acid esters discussed to represent the "physiological substrate" of alkaline phosphatase are glucosephosphate [48] and inorganic pyrophosphate [49], substrates which for this reason were included in our investigation.

Glucose-6-phosphate is not converted by alkaline phosphatase from bovine pulp to a measurable degree (Table III). Thus, the enzyme is clearly different from intestinal alkaline phosphatase, which hydrolyzes this substrate almost as fast as *p*-nitrophenylphosphate [50]. Since our experiments on the substrate specificity of alkaline phosphatase from bovine dentine and enamel showed no reaction with glucosephosphates either (Table IV) it appears that these substrates do not serve as a source of inorganic phosphate for tooth mineralization as discussed earlier [48].

The other hypothesis mentioned above tries to explain the biological function of alkaline phosphatase in bone and teeth with the hydrolysis of inorganic pyrophosphate, a known inhibitor of mineralization [49, 51–55].

Indeed, a considerable proportion of the inorganic pyrophosphatase activity in bovine pulp is inhibited in the presence of levamisole and, therefore is thought to represent a hydrolytic activity of alkaline phosphatase. But to act as a scavenger of pyrophosphate in the mineralization process the enzyme should be detectable at the mineralization front, too. But, despite the fact that high alkaline phosphatase activity is found in enamel and dentine samples with *p*-nitrophenylphosphate, no pyrophosphate hydrolysis is observed in photometric/fluorimetric measurements. The sensitivity of the test systems excluded any inorganic pyrophosphatase activity higher than 10^{-4} U/mg hard tissue. This result proves a considerable difference in substrate specificity of alkaline phosphatase of dental hard tissue as compared to the enzyme found in pulp.

In any case the time-defined appearance of alkaline phosphatase both in pulp and in hard tissue suggests a role of this enzyme in the process of

mineralization. Such a role is also confirmed by a similar decrease in alkaline phosphatase activity observed in human incisors [16].

The negative results observed in the study of dentine samples with different phosphoric acid esters suggest that alkaline phosphatase in dental tissue is a rather specific enzyme but its function is not yet known. The assumption that our tests with dental hard tissue were performed under approximate physiological conditions, with alkaline phosphatase largely remaining membrane-bound in its native environment, is supported by the fact, that addition of detergents did not increase the activity of suspended tissue samples (Table I).

The histological studies also indicate that alkaline phosphatase is related with the mineralization of the dental hard tissue: Contrary to the subodontoblasts no alkaline phosphatase activity was observed in the odontoblasts prior to mineralization. The time dependent appearance and localization of alkaline phosphatase in bovine incisors resembles the odontogenesis of hamster and rat [56, 57]. The previously reported presence of alkaline phosphatase in the odontoblasts during matrix formation [58] could not be confirmed by our study, we think that the former finding is due to diffusion artifacts resulting from the very active subodontoblasts.

B Adenosintriphosphatase

The occurrence of ATPase in mineralizing tissues is relevant because of the release of phosphate and the Ca^{2+}-transport. In accordance with the detection of a Ca^{2+}-activated ATPase in the enamel organ of rats [22, 59] and in the odontoblasts of men and rat [19, 20, 60] we found the enzyme in the pulp and hard tissues of bovine incisors. The enzyme was distinguished from Na^+/K^+-ATPase and other phosphohydrolases by the use of ouabain, levamisole, molybdate and tungstate. The temporal and local appearance of the Ca^{2+}-ATPase is similar to alkaline phosphatase: The reduced activity typical for fully mineralized incisors could point to a specific role of the enzyme in matrix formation or mineralization.

C Acid phosphatase

Acid phosphatases in dental tissues possess a low pH-optimum and poor substrate specificity as do acid phosphatases from other tissues. This reflects their participation at lysosomal processes.

The constancy of the activity is evidence against a specific participation of acid phosphatase in the mineralization process. Further evidence against such a role is the low activity of this isozyme in samples of partially mineralized dentine and enamel.

The differential behaviour of acid phosphatase in the stratum reticulare and in the outer enamel epithelium towards fluoride and tartrate proves the existence of at least two molecular forms of the enzyme. The occurrence of isozymes of acid phosphatase with different inhibition patterns was also observed in the enamel organ of rat molars [17, 61].

The properties of acid phosphatase from bovine pulp and the isozymes from entire toothgerms of rat molars [62] are very similar, as shown in Table XII. Common characteristics are the pH-optimum, a marked inhibition by tartrate, and a less pronounced activation by ascorbic acid. Based on these data both dental isozymes are also clearly distinguishable from rat bone acid phosphatase (Table XII), a tartrate insensitive enzyme form, with considerably higher pH-optimum [62].

Table XII. Comparison of acid phosphatase activity from rat and cattle with *p*-nitrophenylphosphate.

	Rat bone*	Entire molar tooth germ from rat*	Pulp of bovine incisors
pH-Optimum	5.6–6.0	4.6–5.4	4.9
Tartrate inhibition (10 mM)	0%	40%	40%
Residual activity after 60 min at 50 °C	48%	50%	32%
Activation by 5 mM ascorbic acid	5–6 ×	1.6 ×	1.9 ×
Pyrophosphatase activity (tartrate insensitive)	+	+	+

* Lit. cit. [62]; + activity present.

Considering the temporal appearance of the acid phosphatase isozymes, we correlate these enzymes rather with matrix secretion than with mineralization, as discussed earlier [63].

D Inorganic pyrophosphatase

Inorganic pyrophosphatase is an ubiquitous enzyme which is found in all cells of the dental tissue, but little is known about the absolute specificity of the enzyme. Its physiological function is thought to be the hydrolysis of inorganic pyrophosphate, a metabolite of several synthetic pathways. Since inorganic pyrophosphate is also hydrolyzed by unspecific acid and alkaline phosphatases, these enzyme activities were distinguished by applying suitable assay conditions and the inhibitors shown in Table VIII. The results show, that under the conditions used, inorganic pyrophosphate is hydrolyzed by a specific inorganic pyrophosphatase and, to a lesser extend by alkaline phosphatase. The contribution of the latter enzyme activity is represented by the levamisole inhibitable part of pyrophosphate hydrolysis. No interference from acid phosphatase was observed in pyrophosphatase assays: Fluoride and tartrate showed no influence on the relative activity of inorganic pyrophosphatase.

Since highest pyrophosphatase activities were found in the pulps of fully mineralized teeth and virtually no activity at all was observed in the dentine of mineralizing incisors, we rule out a participation of this enzyme in the mineralization process.

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