Electrophoretic separation of alkaline and acid phosphatase isoenzymes from the pulp of monkey teeth

A. FRANZÉN & G. HASSELGREN

Department of Oral Pathology, Karolinska Institute, Stockholm, Sweden, and Department of Endodontics, University of Lund, Malmö, Sweden

Franzén, A. & Hasselgren, G. Electrophoretic separation of alkaline and acid phosphatase isoenzymes from the pulp of monkey teeth. *Acta Odontol. Scand.* 371–375

Monkey pulps were homogenized in a Triton tris solution. After three centrifugation steps (800, 20000, and 105000 *g*) the supernatant was applied on acryl amide columns at pH 7.5 in a tris-diethyl barbituric acid buffer. Electrophoresis was performed at a constant current of 2.5 mA per gel column at 18–20 °C.
Incubations for alkaline phosphatase (E.C. 3.1.3.1) were carried out at pH 8.3 using naphthol-AS-MX-phosphate as substrate and Fast Red Violet LB salt as coupler. Incubations for acid phosphatase (E.C. 3.1.3.2) were undertaken at pH 5.0 using α-naphtyl phosphate as substrate and hexazotized pararosanilin as coupling agent. After the incubations for alkaline phosphatase as well as acid phosphatase two bands showing enzyme activity were demonstrated. By means of treatment with heat (56 °C) prior to incubation or addition of vanadate or pyrophosphate to the incubation medium it was shown that the main part of the fast moving alkaline phosphatase band was sensitive to these procedures. The alkaline phosphatase of the slow moving band appeared to be resistant to heat or the addition of inhibitors.

Key-words: Dental pulp; enzyme histochemistry

Gunnar Hasselgren, Department of Endodontics, School of Dentistry, University of Lund, S-214 21 Malmö, Sweden

Most investigations concerning alkaline and acid phosphatase in dental tissues have been carried out using rodent teeth. A similarity between the phosphatases of hard tissue producing cells in teeth and bone have been found in biochemical and histochemical investigations (5, 6, 7, 13). Recently, two alkaline phosphatase isoenzymes have been demonstrated in the pulp of rat teeth by means of a histochemical technique and the use of certain enzyme inhibitors (10). This finding has been corroborated in a biochemical investigation of developing rat teeth in which two separate bands showing alkaline phosphatase activity could be demonstrated electrophoretically (4).

By means of the same histochemical technique two alkaline phosphatases have also been demonstrated in the pulps of human and monkey teeth (9). However, the distribution patterns of these two isoenzymes were not similar to those which had been observed in the rat. Therefore, the aim of the present study was to separate and characterize alkaline phosphatase isoenzymes from monkey dental pulps by means of electrophoresis and specific inhibitors.

Acid phosphatase has previously been

Received for publication, July 23, 1978

demonstrated by histochemical means in the dental pulp of rat, monkey and man but the use of specific inhibitors did not reveal more than one enzyme (7, 8). Therefore, it was also the intention to investigate if more than one acid phosphatase in the monkey pulp could be demonstrated electrophoretically.

MATERIAL AND METHODS

Tissue preparation: Developing teeth from two-year-old monkeys (Macaca fascicularis) were used in this study. The animals were killed by an overdose of mebumal sodium. The heads were, after decapitation, frozen in hexane cooled with solid CO_2 (−75 °C). Later the jaws, still frozen, were separated from the scull with a saw. The teeth were then split longitudinally and the pulp tissue, including some predentin, was removed by means of a small excavator.

Extraction procedure: The pulp tissue was transferred to a medium (1 % Triton-X-100 in a 0.05 M tris buffer pH 7.4) and homogenized in a Potter-Elvehjem homogenizer. The homogenate was then left on a magnetic stirrer for 30 min. An aliquot of the homogenate was used as a reference standard to estimate the efficiency of the extraction.

Cell debris, and nuclei were removed at two initial low speed centrifugation steps, 800 *g*, 20 min, and 20 000 *g*, 20 min. The remaining supernatant was then centrifuged at 105 000 *g* for 60 min. The supernatant was dialyzed against a 0.05 M tris buffer, pH 7.4, for 72 hours in order to lower the Triton-X-100 concentration from 1 % to approximately 0.2 %. The whole extraction procedure was carried out at 4 °C.

Enzyme assay: The alkaline phosphatase (E.C. 3.1.3.1) activity was determined by using paranitrophenyl phosphate as substrate. The incubations were carried out at pH 8.3 in a 0.05 M tris buffer and at pH 10.2 in a sodium bicarbonate buffer (3) and magnesium chloride (1mM) was added to the incubation media (6).

The acid phosphatase (E.C. 3.1.3.2) activity was determined by using paranitrophenyl phosphate as substrate in 0.1 M acetate buffer at pH 5.0.

All incubations were carried out at 37 °C for 15 min. The enzyme reactions were stopped by the addition of 0.1 M NaOH to the incubation media. The amount of liberated paranitrophenol was determined at 410 nm in a spectrophotometer.

Electrophoresis: The electrophoretic procedure was done on 7.5 % acryl amide columns at pH 7.5 in a tris-diethylbarbituric acid buffer (14).

The electrophoresis was carried out at a constant current of 2.5 mA per gel column at 18–20 °C. Bromphenol blue was used to stain the albumin in the migration front and the electrophoresis was stopped when this front had reached a distance of 2–3 mm from the lower gel border. The gels were then removed from the glass tubes and incubated for demonstration of acid and alkaline phosphatase activity or stained for demonstration of proteins. The incubations for alkaline phosphatase activity were performed at pH 8.3 using naphthol-AS-MX-phosphate as substrate and Fast Red Violet LB salt as coupler (2). Incubations were also carried out with 5 mM sodium metavanadate or 100 mM sodium pyrophosphate added to the incubation medium. Before incubation some gel columns were kept at 56 °C for 15–30 min. The incubations for acid phosphatase were done at pH 5.0 using α-naphthyl phosphate as substrate and hexazotized pararosanilin as coupling agent (1, 12). Control incubations without substrate were also performed.

The proteins were stained with 0.1 % Comassie Brilliant blue G-250 for 12 hours at room temperature. The gels were destained in 7.5 % acetic acid until the background was unstained.

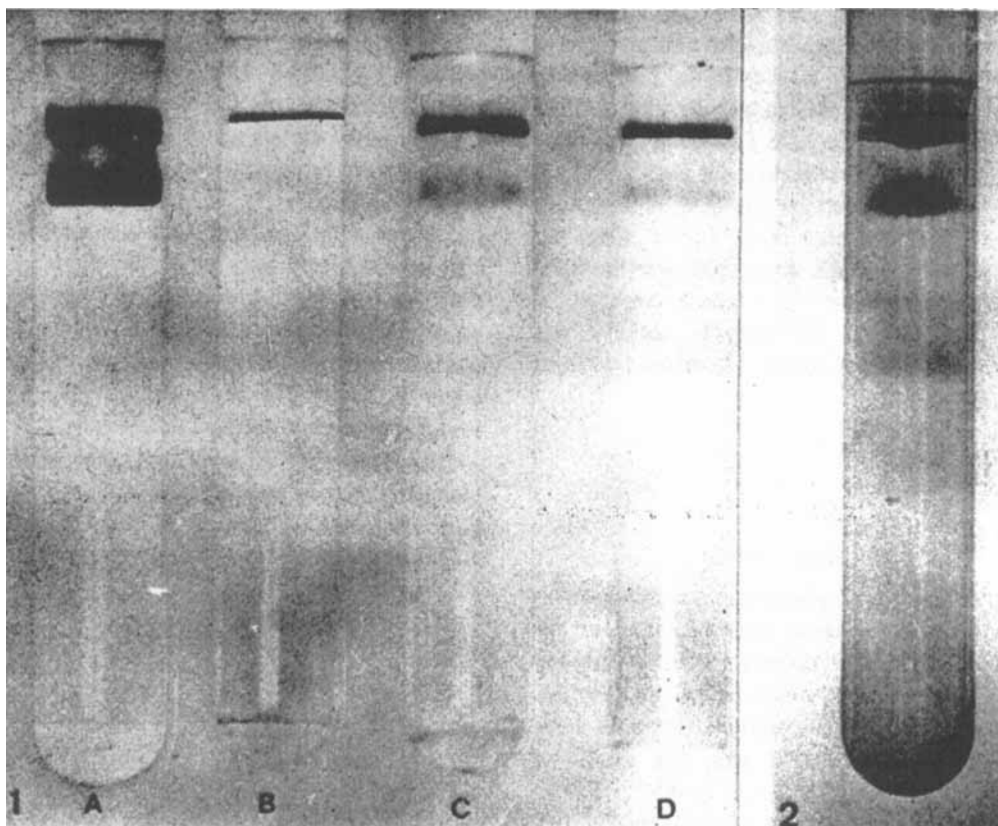

Fig. 1. Acryl amide gel columns incubated for demonstration of alkaline phosphatase at pH 8.3 using naphthol-AS-MX-phosphate as substrate and Fast Red Violet LB salt as coupler after electrophoresis of extract from monkey pulps.

A. Control. There are two bands showing alkaline phosphatase activity.
B. Sodium metavanadate (5 mM) has been added to the incubation medium. There is an almost total inhibition of the enzyme activity in the fast moving band and a decrease in activity in the slow moving band.
C. Pretreatment with heat (56 °C) for 30 min prior to incubation. There is a markedly decreased enzyme activity, especially in the fast moving band.
D. Sodium pyrophosphate (100 mM) has been added to the incubation medium. There is a markedly decreased enzyme activity, especially in the fast moving band.

Fig. 2. Acryl amide gel column incubated for demonstration of acid phosphatase at pH 5.0 using α-naphthyl phosphate as substrate and hexazotized pararosanilin as coupler after electrophoresis of extract from monkey pulps. There are two bands showing acid phosphatase activity.

RESULTS

The activities of alkaline and acid phosphatases in the high speed supernatant exceeded 90 % of the activities in the original homogenates. In the gel columns which had been incubated for demonstration of alkaline phosphatase two bands were demonstrated (Fig. 1). When sodium metavanadate was added to the incubation medium the activity in the fast moving band was almost completely inhibited (Fig. 1). In these instances the slow moving band appeared to be thinner as compared to the gel columns incubated without the addition of an inhibitor.

Pretreatment with heat (56 °C) before incubation for alkaline phosphatase gave a markedly weaker activity in the fast moving band. A similar pattern was also found when sodium pyrophosphate (100 mM) had been added to the incubation medium.

The investigations for acid phosphatase also demonstrated two bands (Fig. 2) showing the same electrophoretic mobility as the two alkaline phosphatase bands.

No staining for enzyme activity was found in the gel columns incubated without substrates.

DISCUSSION

After the electrophoresis two bands showing alkaline phosphatase activity could be demonstrated. The activity in the fast moving band was almost inhibited by vanadate and it was sensitive to pretreatment with heat. This is in agreement with the results of Franzén et al. (4).

It has been shown histochemically that heat, vanadate and phosphate affect the alkaline phosphatase in the odontoblasts and in the pulpal connective tissue of primate teeth (9). Thus it appears likely that the fast moving band contains mainly alkaline phosphatase from these parts of the pulps.

In agreement with previous results (4) the slow moving band on the whole was unaffected by pretreatment with heat or the inhibitors which were added to the incubation medium. By histochemical means this resistant type of isoenzyme in the rat dental pulp was observed only in the subodontoblastic cell layer (4). This isoenzyme was also demonstrated in the subodontoblastic cell layer in human and monkey pulps but in these instances the enzyme was also observed in the odontoblasts and in the pulpal connective tissue (9). Although a species difference has been noted by histochemical means between primate and rat teeth concerning the distribution pattern of the two alkaline phosphatases, the results of the present study and the previous electrophoretical study of rat dental pulps (4) suggest that the same isoenzymes were demonstrated in pulpal homogenates from monkey and rat.

The incubations for acid phosphatase also demonstrated two bands. So far it has not been possible in histochemical studies to do selective inhibition of pulpal acid phosphatase, and it is therefore not possible to relate the separate bands in the gel columns to different cell types.

The fact that alkaline and acid phosphatase appeared to have the same electrophoretic mobility may suggest that the same enzymes are active both at an acid and an alkaline pH.

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