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FRACTIONATION OF ENZYMES FROM HUMAN DENTAL PLAQUE HYDROLYSING P-NITROPHENYL PHOSPHATE

by

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INTRODUCTION

It has been shown that certain proteolytic enzymes are capable of liberating phosphate from human dental enamel (*Paunio et al.*, 1968), and it was discussed that enzymes could be a primary factor in the degradation of dental enamel and dentine in carious processes. One interesting example of enzyme activity, obtained in preliminary and so far unpublished studies with model catalysts, was the enzymic hydrolysis of p-nitrophenyl phosphate. This part of the study deals with the demonstration and the fractionation of this particular enzyme activity in human dental plaque material.

MATERIALS AND METHODS

Chemicals

All chemicals used in the study were obtained from E. Merck AG (Darmstadt, Germany) if not otherwise stated.

Substrate

Throughout the study p-nitrophenyl phosphate was used, obtained from Sigma Chemical Company (St. Louis, Mo., U.S.A.).

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Enzyme preparation from plaque material

The enzyme preparation originated from plaque obtained from patients having a high number of carious lesions. The dental plaque material was collected into cold (+4°C) 0.154 M NaCl solution. The saline solution with the plaque material was then immediately centrifuged for 20 min at 20.000 ×g at +4°C (Sorvall, Superspeed Centrifuge, RC-2B). The pellicle was discarded and the supernatant was collected. The enzyme preparations thus obtained from different patients were then pooled.

Determination of enzyme activity

The method was based on measurement of the color intensity produced by the enzymatically liberated yellow p-nitrophenol anion at 410 mμ (Hitachi-Perkin Elmer UV—VIS spectrophotometer). The procedure used was the following: from 10^{-3} M substrate stock solution was 0.1 ml taken into the reaction mixture. The reaction mixture contained in addition of 0.3 ml buffer, 0.1 ml water or other compounds to be studied, and finally of 0.1 ml enzyme solution. The test tubes with the reaction mixture were then incubated at 37°C for different length of time. After the incubation the reaction mixtures were placed in an iced water bath, followed by an addition of 1 ml 0.2 M NaOH.

The effect of pH on the rate of hydrolysis of p-nitrophenyl phosphate

The following buffer solutions covering a pH range from 3.4 to 12, were used: ββ-dimethylglutaric acid-NaOH buffer, glycine-NaOH buffer, and boric acid-borax buffer. The enzyme solutions were added to the incubation mixture as in usual activity assays consisting of buffer solutions of different pH values.

The stability of the enzyme preparation to various pH changes

The occurrence of irreversible destruction of the enzyme preparation studied was tested by exposing the enzyme preparation to a range of pH values at 37°C for 20 min, followed then by an activity assay of the enzymes after adjustment of the pH value to the pH optimum value for the tested substrate. The method is principally the same as described by *Mäkinen* (1969), plaque enzyme preparation was first dialysed against a twentyfold volume of water for 24 hours in order to remove excess buffer. Enzyme dilutions, having different pH values, were prepared by pipetting 1 μl of undiluted enzyme

solution at the bottom of a small test tube. Thereafter 100 μl of 0.0014 M universal buffer (*Britton & Wellford*, 1937) of varying pH values was added. One μl enzyme solution was then discharged from the test tubes by means of one microliter syringe equipped with 0.01 μl graduations (Shandon Scientific Company Ltd, London, England). The enzyme solutions were then incubated at 37°C for 20 min in tubes sealed with parafilm. After the incubation, the other components of the usual reaction mixture (0.3 ml of universal buffer, 0.1 ml substrate solution, and 0.1 ml water) were added. The buffer now used had a molarity of 0.056 in the reaction mixture, and the substrate a molarity of 1.66×10^{-4}, which was found to saturate the enzyme at all pH values studied, and which consequently eliminated the effect of pH on the affinity. The reaction mixtures were then again incubated at 37°C for different length of time given in more details in the results. The enzyme activity was determined in the usual way described earlier in this paper. At the same time usual pH dependence curve was determined as earlier in a reaction mixture containing 0.3 ml 0.0014 M universal buffer, 0.1 ml 1.66×10^{-4} M substrate, 0.1 ml water, and 0.1 ml enzyme solution.

Gel filtration

Pooled enzyme solution was freeze dried to reduce the original volume to one tenth. The concentrated enzyme solution was then gel filtered through Sephadex® G-100 columns (Sephadex G-100 fine, supplied by Pharmacia Fine Chemicals, Uppsala, Sweden). The gel filtration as well as other chromatographical steps were performed at +2°C. The enzymes were recovered by assaying the enzyme activity as described earlier in this study.

Desalting of the enzyme preparation by using Sephadex G-25 gel filtration

The pooled enzyme solutions after Sephadex G-100 gel filtration were transfered into 0.005 M $\beta\beta$-dimethylglutaric acid buffer, pH 6.6, by gel filtration through Sephadex G-25 13 cm × 2 cm columns (Sephadex G-25 fine, supplied by Pharmacia Fine Chemicals).

Column chromatography on substituted cellulose

DEAE-cellulose (Carl Schleicher & Schüll, Dassel/Kr. Einbeck, Germany) with the particle size 200—230 mesh was used in these experiments. Different fractionations were performed at different pH values to search for the optimal conditions for the fractionation of the enzymes.

RESULTS

Determining of pH optimum for the rate of hydrolysis of p-nitrophenyl phosphate with crude enzyme preparation

These experiments revealed when tested in 0.02 M boric acid-borax buffer that the rate of hydrolysis was greatest at the pH value slightly less than 8 with the crude enzyme preparation (crude plaque enzyme extract).

Sephadex G-100 gel filtration

Fig. 1 reveals the distribution of the p-nitrophenyl phosphate hydrolysing enzymes of dental plaque showing three apparent enzyme peaks. Three enzyme pools were prepared from activity peaks by pooling the active portions for further fractionation and studies.

The stability of the enzyme preparations to various pH changes

The enzyme pools derived after Sephadex G-100 gel filtration were subjected to pH stability tests. The results obtained in these experiments are seen in

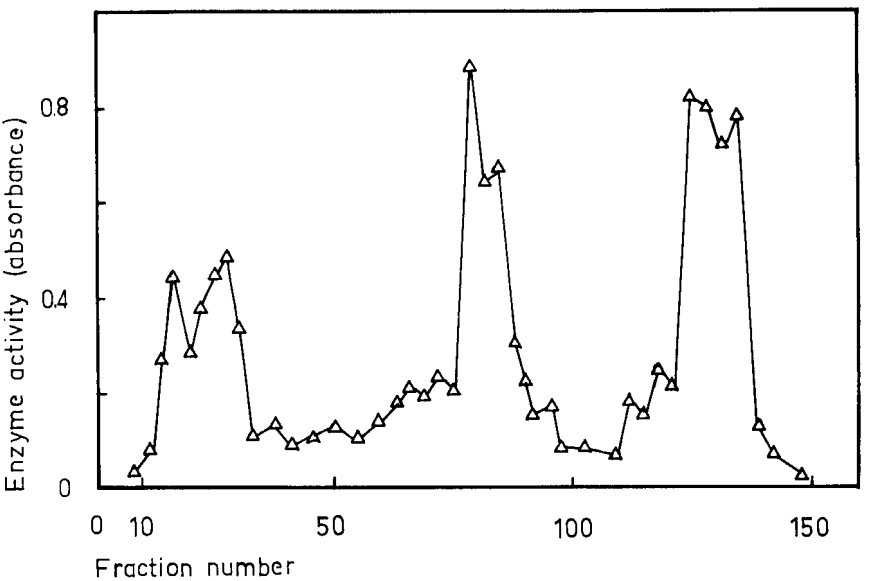

Fig. 1. Sephadex G-100 gel filtration of human dental plaque enzymes capable of hydrolysing p-nitrophenyl phosphate. Column: 85 cm × 2.8 cm; hydrostatic pressure 20 cm; fraction size collected 2 ml; flow rate 3 ml/h; buffer 0.05 M $\beta\beta$-dimethylglutaric acid buffer pH 7.0; temperature +2°C; sample applied to the column 5 ml enzyme solution.

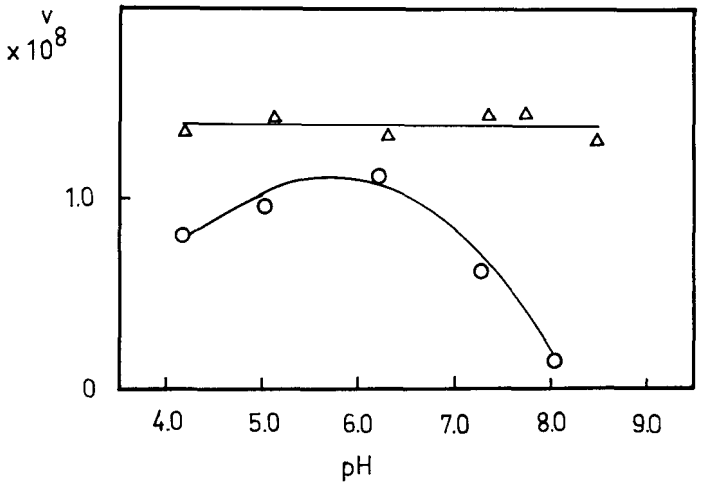

Fig. 2. The stability of the enzyme preparation towards pH changes of Pool I (△). Detailed method described in the text. The rate of hydrolysis (v) is expressed as the molar change of liberated p-nitrophenol in the reaction mixture per min. Simultaneously performed pH dependence curve of the rate hydrolysis of p-nitrophenyl phosphate (o) by the same enzyme preparation.

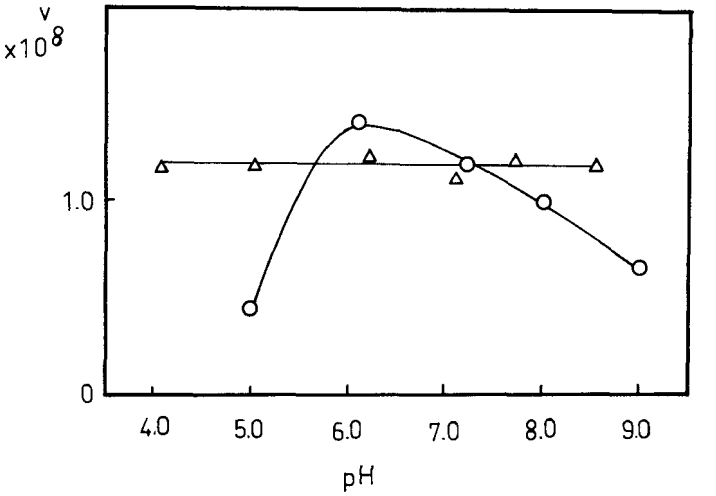

Fig. 3. The stability of the enzyme preparation Pool II (△) towards pH changes. Detailed method described in text. The rate of hydrolysis (v) is expressed as the molar change of liberated p-nitrophenol in the reaction mixture per min. Simultaneously performed pH dependence curve of the rate of hydrolysis of p-nitrophenyl phosphate (o) by the same enzyme preparation.

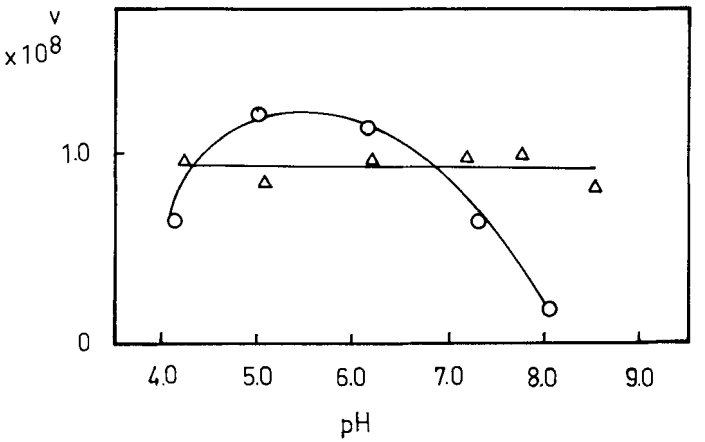

Fig. 4. The stability of enzyme preparation (Pool III) towards pH changes (△). Detailed method described in text. The rate of hydrolysis is expressed as the molar change of liberated p-nitrophenol in the reaction mixture per min. Simultaneously performed pH dependence curve of the rate of hydrolysis of p-nitrophenyl phosphate (o) performed on the same preparation.

Figs. 2, 3, and 4. Pool I showed a firm stability over the tested pH values. No distinction in the rate of hydrolysis of p-nitrophenyl phosphate could be noted. The same trend could be noted when enzymes from the other to pools were tested. These enzymes showed a similar pH stability over the tested pH values. On the other hand, when the experiments were performed in universal buffer the pH dependence curve revealed that the rate of hydrolysis was highest at the values below pH 7.

DEAE-cellulose fractionation of the pooled enzyme preparations after Sephadex G-100 gel filtration

The distribution of the enzymes of Pool I, transfered into 0.005 M $\beta\beta$-dimethylglutaric acid through Sephadex G-25 gel filtration, can be seen in Fig. 5 showing two apparent enzyme peaks with p-nitrophenyl phosphate as substrate. The second peak was excluded from the column at low NaCl concentration in the elution buffer. Analogous results were obtained with the corresponding enzymes of Pool II and III showing two apparent enzyme peaks after the ionexchange chromatography on DEAE-cellulose (Figs. 6 and 7). The second peak of Pool II was excluded from the column by about 0.3 M NaCl concentration in the elution buffer. In the same way, the second

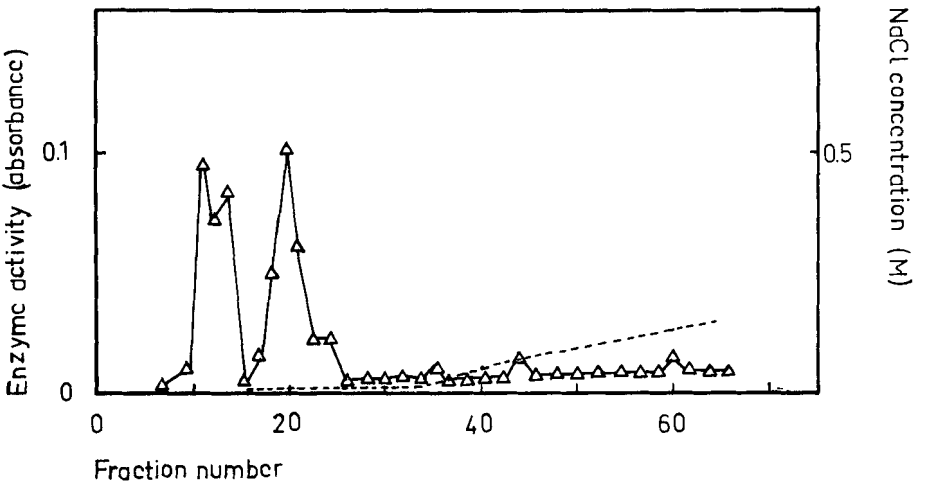

Fig. 5. DEAE-cellulose chromatography performed on the enzyme solution derived from Pool I formed after Sephadex G-100 gel filtration. Column: 10 cm×2 cm; hydrostatic pressure 150 cm; fraction size collected 2 ml; buffer 0.005 M $\beta\beta$-dimethylglutaric acid, pH 6.6, with an addition of a linear salt gradiation with the same buffer containing 1 M NaCl; sample solution transfered into 0.005 M $\beta\beta$-dimethylglutaric acid buffer, pH 6.6, by gel filtering through Sephadex G-25.

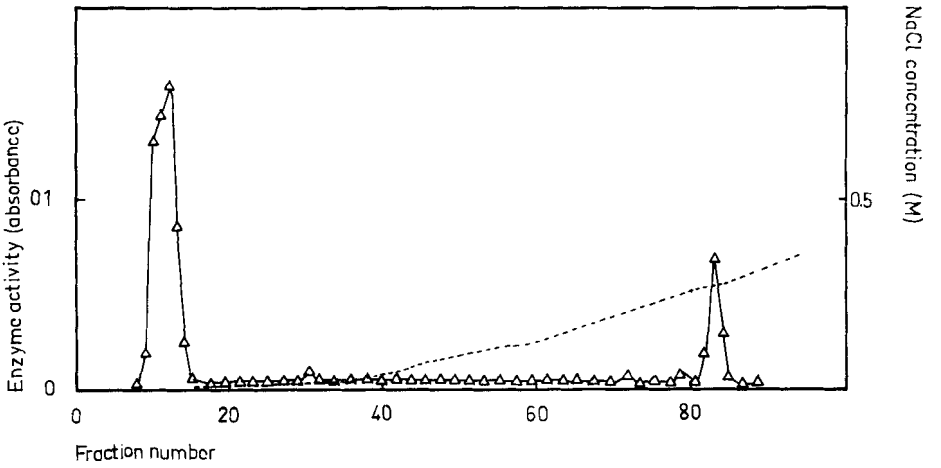

Fig. 6. DEAE-cellulose chromatography performed on the enzyme derived from Pool II formed after Sephadex G-100 gel filtration. Column: 10 cm×2 cm; hydrostatic pressure 150 cm; fraction size collected 2 ml; buffer 0.005 M $\beta\beta$-dimethylglutaric acid pH 6.6, with an addition of a linear salt gradiation with the same buffer containing 1 M NaCl: sample enzyme solution transfered into 0.005 M $\beta\beta$-dimethylglutaric acid buffer, pH 6.6 by gel filtering through Sephadex G-25.

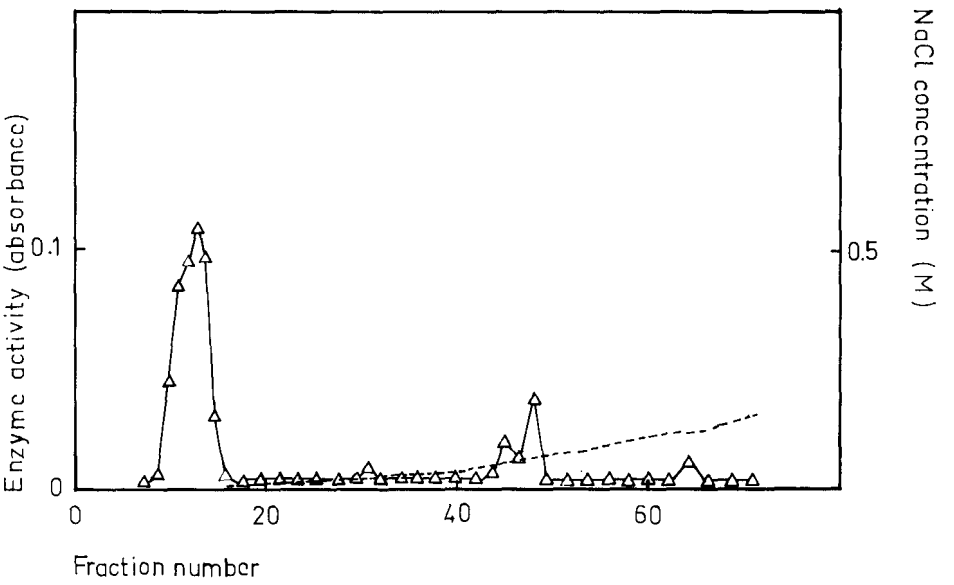

Fig. 7. DEAE-cellulose chromatography performed on the enzyme derived from Pool III formed after Sephadex G-100 gel filtration. Column: 10 cm×2 cm; hydrostatic pressure 150 cm; fraction size collected 2 ml; buffer 0.005 M $\beta\beta$-dimethylglutaric acid, pH 6.6, with an addition of a linear salt gradient with same buffer containing 1 M NaCl: sample enzyme solution transfered into 0.005 M $\beta\beta$-dimethylglutaric acid buffer, pH 6.6, by gel filtering through Sephadex G-25.

enzyme peak of Pool III was excluded from the column by slightly less than 0.1 M NaCl concentration in the elution buffer. The fractionations performed in the same way but using different pH values of 0.005 M $\beta\beta$-dimethylglutaric acid buffer (pH 5.5 and 8.0) showed no significant improvement of the fractionation of these enzymes.

DISCUSSION

The use of synthetic compounds as model substrates in enzyme studies is determined by the knowledge of their structure, which is not the case with most »natural substrates» being used in earlier investigations. Further, the knowledge of the chemical structure of the substrate allows conclusions to be drawn on the effect of different factors affecting a specific enzyme activity; in this study the enzyme hydrolysis of the well known aster linkage between phosphate and p-nitrophenol.

Studies with crude enzyme preparations does not give valid results of the enzymic activity being studied, because these contain in addition to enzymes other components (organic acids etc.), which may interfere with the enzyme reactions, and they may even catalyse the hydrolysis of the substrates. A purification of the crude enzyme preparation must be undertaken in order to remove these adventitious compounds before chemical studies can be undertaken. In this study a fractionation and purification of the enzymes having a p-nitrophenyl phosphate hydrolysing activity was performed. After both Sephadex G-100 gel filtration and DEAE-cellulose ionexchange chromatography 6 different enzyme preparations were obtained, all having the same enzyme activity. These fractionation experiments suggest an existence of several enzymes having this particular phosphorolytic activity. This is supported by the different K_d values (Fig. 1), obtained for the different pooled enzymes after Sephadex G-100 gel filtration, and by the different chemical behaviour of these enzymes in the DEAE-cellulose ionexchange chromatography.

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SUMMARY

Human dental plaque material was collected into 0.154 M NaCl solution and centrifuged. The enzymes dissolved in the saline solution were then fractionated according to their ability to hydrolyse p-nitrophenyl phosphate, used in this study as the working substrate. The fractionation was performed by gel filtration through Sephadex G-100 and on DEAE-cellulose ionexchange chromatography. The results revealed the existence of several enzymes in human dental plaque possessing this activity. The enzymes responsible for this activity showed also a firm stability towards pH changes in the surrounding media.

RÉSUMÉ

FRACTIONNEMENT DES ENZYMES PROVENANT DE LA PLAQUE BACTÉRIENNE DENTAIRE HUMAINE ET SUSCEPTIBLES D'HYDROLYSER LE PHOSPHATE DE P-NITROPHÉNYLE

Des prélèvements de plaque bactérienne dentaire faits sur des patients venus consulter la clinique dentaire ont été recueillis dans une solution de NaCl 0,154 M et centrifugés. Les enzymes dissous dans la solution saline ont ensuite

été fractionnés suivant leur aptitude à hydrolyser le phosphate de p-nitrophényle, utilis dans ce travail comme substrat. Le fractionnement a été effectué par filtration sur gel à travers le Séphadex G-100 et par fractionnement sur DEAE-cellulose.

Les résultats révélaient l'existence de plusieurs enzymes (au moins 4) possédant cette activité. Ces activités présentaient aussi une forte stabilité à l'égard des changements de pH dans le milieu environnant.

ZUSAMMENFASSUNG

FRAKTIONIERUNG DER p-NITROPHENYLPHOSPHAT HYDROLISIERENDEN ENZYME IN DER MENSCHLICHEN PLAQUE

Menschliche Plaque wurde in eine 0.154 M NaCl-Lösung gesammelt und die Mischung danach zentrifugiert. Die gelösten Enzyme wurden nach ihren p-Nitrophenylphosphat spaltenden Eigenschaften fraktioniert. Die Fraktionierung wurde mit Hilfe von Sephadex G-100 Gelfiltration sowie von DEAE-Zelluloseionenaustauschchromatographie durchgeführt. Aus den Resultaten ergab sich, dass in der Plaque viele Enzyme vorhanden sind, welche jene Aktivität besitzen. Die Enzymaktivitäten erwiesen sich als empfindlich gegen pH-Wechsel.

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