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FRACTIONATION AND SOME PROPERTIES OF ENZYMES FROM HUMAN DENTAL PLAQUE HYDROLYSING N-L-PHENYLALANYL-2-NAPHTHYLAMINE

by

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INTRODUCTION

In previous publication (*Paunio et al.*, 1968) it was shown that enzymes were capable in liberating phosphate from human dental enamel. In these publication it has been discussed that enzymes could be a primary factor in the degradation of dental enamel and dentine in carious processes. The hydrolysis of *N*-L-phenylalanyl-2-naphthylamine was one of the interesting degradation processes obtained in preliminary unpublished studies performed with model catalysts at our laboratory. This study is dealing with the demonstration of this enzyme activity in the plaque, a fractionation of crude plaque material into components, capable in hydrolysing-*N*-L-phenylalanyl-2-naphthylamine, and a partial characterization of enzymes involved.

MATERIALS AND METHODS

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

Substrate. Throughout the study *N*-L-phenylalanyl-2-naphthylamine was used. In some experiments *N*-L-prolyl-2-naphthylamine was also used. The substrates were supplied by Mann Research Laboratories Inc. (New York, N.Y., U.S.A.).

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Enzyme preparation. Plaque material was collected from different persons coming to the clinic. The plaque was collected into iced 0.154 M NaCl solution. The saline plaque mixture was then cold (+3°C) centrifuged for 20 minutes at 20.000 × g (Sorvall model RC-2B). The supernatant obtained was collected and the pellicle was discarded. Different enzyme preparations obtained from different persons were then pooled.

Determination of the hydrolysis of N-L-phenylalanyl-2-naphthylamine and N-L-prolyl-2-naphthylamine. The method was based on measurement of the color intensity produced by diazonium salt coupling of enzymatically liberated 2-naphthylamine. The procedure used has been described earlier (*Mäkinen*, 1969). Color absorbance was read at 525 mμ. Hitachi-Perkin Elmer UV-VIS Spectrophotometer was used throughout the study. In the experiments performed with certain enzyme affectors the liberated 2-naphthylamine was was coupled by the Bratton-Marshall reaction (1939).

The effect of pH on the hydrolysis of N-L-phenylalanyl-2-naphthylamine. Following buffer solutions were used covering a pH range from 3.6 to 12: β,β-dimethylglutaric acid-NaOH, glycine-NaOH, and boric acid-borax. The enzyme solution was added to the incubation mixture as in usual activity assays consisting of buffer solutions of different pH values.

Gel filtration. Pooled enzyme solution was freeze dried to reduce the original volume to the 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 enzymes were recovered by assaying the enzyme activity with *N*-L-phenylalanyl-2-naphthylamine as substrate as described earlier in this study.

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 30 min, followed then by a testing of the activity after adjustment of the pH values to the pH optimum value of the tested substrate. The method is principally the same as described by *Mäkinen* (1969) for rat liver aminopeptidase B and is the following: plaque enzyme preparation was first dialysed against a twenty fold 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 to the bottom of a 6 ml test tube. Thereafter 100 μl of 0.0014 M universal buffer (*Britton & Wellford*, 1937) was added of varying pH values. One μl enzyme solu tion was then discharged on the bottom of the test tube by means of one microliter syringe equipped with 0.01 μl graduation. (Shandon Scientific Company Ltd, London, England). The enzyme solutions were then incu-

bated at 37°C for 30 minutes in tubes sealed with parafilm. After the incubation, the other components of the usual reaction mixture (0.3 ml of 0.05 M β,β-dimethylglutaric acid buffer, 0.5 ml substrated solution, and 0.1 ml water) were added. 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 elsewere in the paper. The experiments were performed separately in two sets of experiments with different substrate concentrations also given in the results. At the same time the usual pH dependence curve was determined as described earlier in reaction mixture containing 0.05 M β,β-dimethylglutaric acid buffer of different pH values (also shown in the results), 0.1 ml substrate, 0.1 ml water, and 0.1 ml enzyme solution. Corresponding experiments were performed with both *N*-L-phenylalanyl-2-naphthylamine and *N*-L-prolyl-2-naphthyla mine as substrates.

The effect of various compounds on the rate of hydrolysis of N-L-phenylalanyl-2-naphthylamine. The following compounds were studied (given with their abbreviations and sources): dithiotreitol, DTT (Calbiochem, Los Angeles, California, U.S.A.); 1-1-tosylamido-2-phenylethylchloromethyl ketone, TPCK, (Mann Research Laboratories); phenylmethanesulphonyl fluoride, PMSF (Mann Research Laboratories); N-ethylmaleimide, NEM (Mann Research Laboratories); diphenylcarbamyl chloride, DPCC (Mann Research Laboratories); and L-cysteine-HCl (Sigma Chemical Company, St. Louis, Mo., U.S.A.). In addition to these compounds $CaCl_2$, $MgCl_2$, $CuCl_2$, NaF, $MnCl_2$, and Na_2EDTA were tested. The compounds were dissolved in the incubation buffer to form a 10^{-2} M stock solutions, except for DPCC which was dissolved in acetone to form a 10^{-2} M solution. The enzyme assays were carried out as described earlier, but now in presence of an increasing concentration of the compound to be studied. The concentration of these compounds in the reaction mixtures is given in the results. In the affector experiments performed with PMSF liberated 2-naphthylamine was coupled by the Bratton-Marshall reaction. Appropriate blanks were allways included, for instance to rule out a possible effect of the compounds on the substrate.

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 β,β-dimethylglutaric acid buffer, pH 6.6, by gel filtration through Sephadex G-25 13 cm × 2 cm columns (Sephadex G-25, fine; Pharmacia Fine Chemicals).

Column chromatography on substituted celluloses. DEAE-cellulose (Carl Schleicher & Schüll, Dassel / Kr. Einbeck, Germany) with the particle size

200—230 mesh was used in these experiments. All fractionations were performed at +2°C. Different fractionations were performed at different pH values to search for the optimal conditions for the fractionation of the enzymes.

Determination of the inhibition constant Ki. The apparent inhibition constant K_i was determined according to the plotting method described by Dixon (1953). In this method $1/v_i$ (v_i = rate of the reaction in the presence of the inhibitor) is plotted as the function of the inhibitor concentration (I).

Determination of sodium. The NaCl concentration in the elution buffer of the DEAE-cellulose ionexchange chromatograph was determined by measuring the amount of sodium in the collected fractions. This was performed using a standard method for sodium determination applied atomic absorption spectrophotometers (Perkin Elmer Manual). Perkin-Elmer Atomic Absorption Spectrophotometer, Model 303, was used.

RESULTS

Determination of pH optimum for the hydrolysis of N-L-phenylalanyl-2-naphthylamine with crude enzyme preparation. The effect of pH on the rate of hydrolysis of *N*-L-phenylalanyl-2-naphthylamine was tested in 0.05 M β,β-dimethylglutaric acid buffer of different pH values. The experiments revealed that the most favorable pH value was located around pH 6.6 with the crude enzyme preparation.

Sephadex G-100 gel filtration. Figure 1 reveals the distribution of the enzyme activity (hydrolysis of *N*-L-phenylalanyl-2-naphthylamine), giving three activity peaks when the fractionation was performed in 0.05 M β,β-dimethylglutaric acid buffer, pH 7.0. Three enzyme preparations were prepared from the different activity peaks by pooling (which fractions were pooled is indicated in Figures).

The stability of the enzyme preparation towards pH changes. The results obtained on the stability of the enzyme preparation, pooled after Sephadex G-100 gel filtration, can be seen in Figure 2. The alteration of pH causes lowering of the tested enzyme activities of the enzymes derived from all the enzyme preparations when tested at two substrate concentration levels, resulting in a lowered rate of hydrolysis of the substrates both above and under the pH optimum values. Figure 3 showes the results obtained from corresponding experiments performed with *N*-L-prolyl-2-naphthylamine. It is apparent that no lowering of the enzyme activity could be obtained. The enzymes of both Pool II and III were not able to hydrolyse this substrate

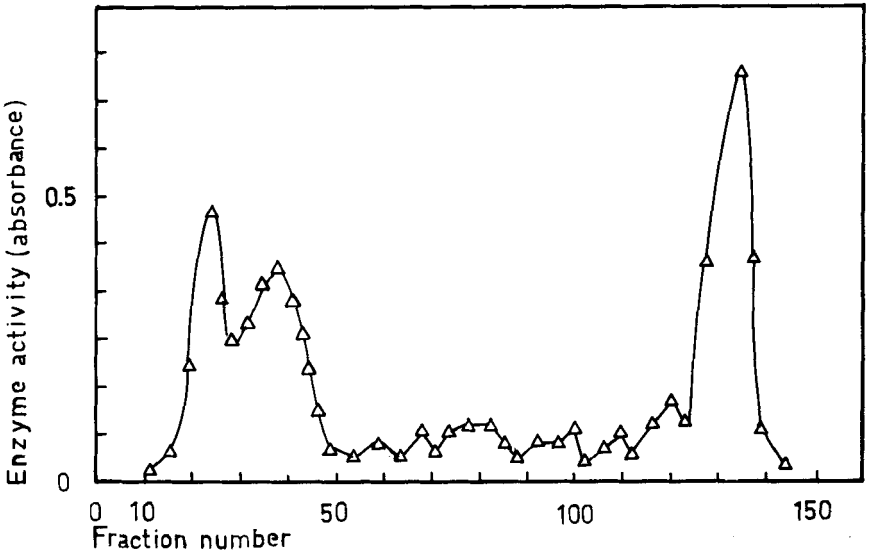

Fig. 1. Sephadex G-100 gel filtration of human dental plaque enzymes capable of hydrolysing *N*-L-phenylalanyl-2-naphthylamine. Column: 85 cm × 2.8 cm; hydrostatic pressure 20 cm; fraction size collected 2 ml; flow rate 3 ml/h; buffer 0.05 M β, β-dimethylglutaric acid pH 7.0; temperature +2°C; sample applied to the column: 5 ml enzyme solution. The fractions from 15 and 30 were pooled to obtain Pool I, and the fractions from 35—50 were pooled to obtain Pool II, and the fractions from 125 and 140 were pooled to obtain Pool III.

at all. The pH dependence curve performed with *N*-L-prolyl-2-naphthylamine as substrate (Fig. 3) showes an unusual distribution of the experimental points, resulting in a flattened pH curve when tested in 0.05 M β,β-dimethylglutaric acid buffer.

The effect of various compounds on the enzyme activities. Of the many compounds tested only some affected the enzymes. Ca^{2+} ions were the only ions capable in activating the enzymic hydrolysis of *N*-L-phenylalanyl-2-naphthylamine by the enzymes of Pool I and III. The enzymes of Pool II were on the other hand inactivated by these ions (Fig. 4 B). Cysteine did inactivate the enzymes of Pool II leaving the enzymes of the Pools I and III unaffected (Fig. 4 C). EDTA ions showed an similar effect, Pool I and II were inactivated by this affector leaving the enzymes of Pool III unaffected (Fig. 4 D). Figure 4 A shows the results of the inhibitory effect of Mn^{2+}, Zn^{2+}, and Cu^{2+} ions on the enzymes derived from Pool II. Cu^{2+} ions were the strongest inhibitors, demonstrated in the Figure, only by two experimental points because of the selected ion concentrations in the

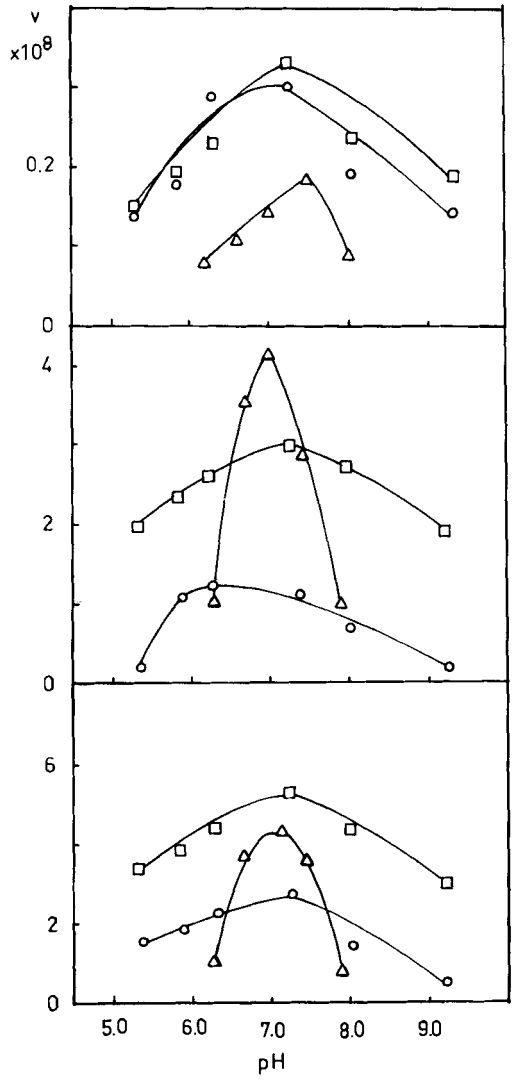

Fig. 2. The stability of the enzymes of Pool I (A), Pool II (B), and Pool III (C) towards pH changes. Detailed method is described in text. The rate of hydrolysis (v) of *N*-L-phenylalanyl-2-naphthylamine, tested at two different substrate concentration levels (0.34×10^{-3} M, ±; and 0.67×10^{-5} M, □), is expressed as the molar change of liberated 2-napthylamine in the reaction mixture per minute. Usual pH dependence curves (△) were performed in 0.05 M β, β-dimethylglutaric acid buffer and final 0.83×10^{-3} M substrate concentration. Incubation time 22 hours.

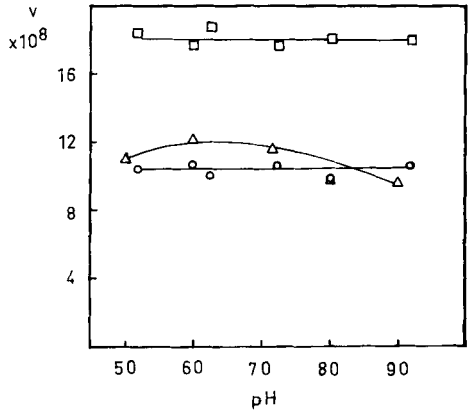

Fig. 3. The stability of the enzymes of Pool I towards pH changes. Detailed method is described in text. The rate of hydrolysis (v) of *N*-L-prolyl-2-napthylamine, tested at two different substrate concentration levels (0.83×10^{-3}, □; and 0.83×10^{-4} M, ±), is expressed as the molar change of liberated 2-napthylamine per minute. Usual pH dependence curve (△) was performed in 0.05 M *β*, *β*-dimethylglutaric acid buffer and a final 0.83×10^{-3} M substrate concentration. Incubation time 4 hours.

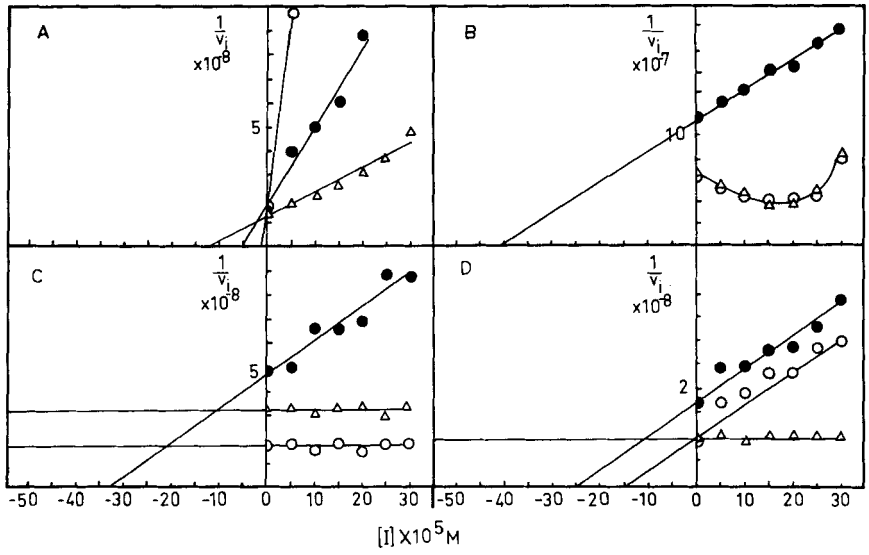

Fig. 4. A. The effect of Cu^{2+} (○), Zn^{2+} (●), and Mn^{2+} (△) ions on the rate of hydrolysis of *N*-L-phenylalanyl-2-naphthylamine by the enzymes of Pool II. Incubation time 16 hours.
B. The effect of Ca^{2+} ions on the rate of hydrolysis of *N*-L-phenylalanyl-2-naphthylamine by the enzymes of Pool I (○), Pool II (○), and Pool III (△). Incubation time 19 hours.
C. The effect of EDTA on the rate of hydrolysis of *N*-L-phenylalanyl-2-naphthylamine by the enzymes of Pool I (○), Pool II (●), and Pool III (△). Incubation time 19 hours.
D. The effect of L-cysteine on the rate of hydrolysis of *N*-L-phenylalanyl-2-naphthylamine by the enzymes of Pool I (○), Pool II (●), and Pool III (△). Incubation time 16 hours.

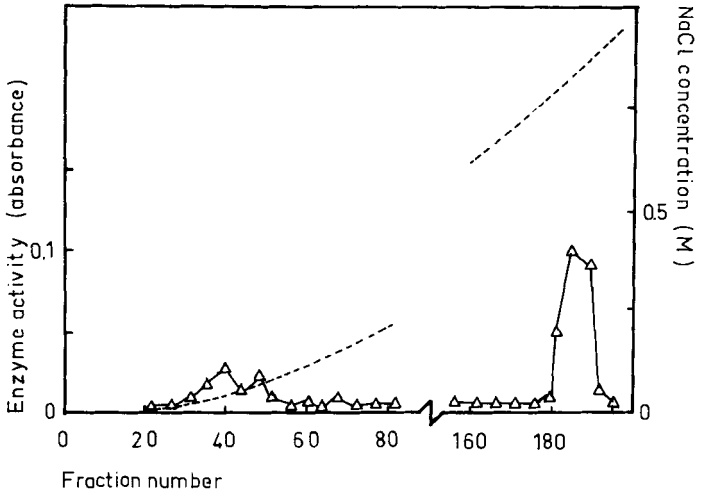

Fig. 5. DEAE-cellulose chromatography performed on the enzyme solution derived from Pool I, obtained after Sephadex G-100 gel filtration. Column: 10 cm × 2 cm; hydrostatic pressure 150 cm; fraction size collected 2 ml; buffer 0.005 M β, β-dimethylglutaric acid pH 6.6, with an addition of a linear salt gratiation (0—1 M) with the same buffer containing 1 M NaCl; sample, enzyme solution transfered into 0.005 M β, β-dimethylglutaric acid buffer, pH 6.6, by gel filtering through Sephadex G-25.

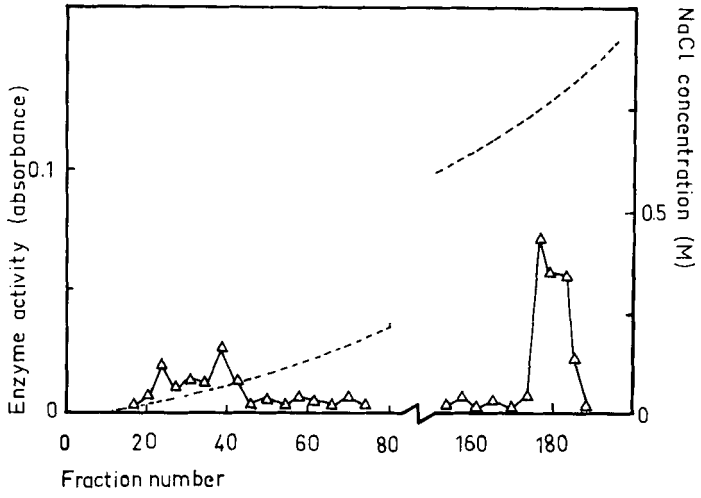

Fig. 6. DEAE-cellulose chromatography performed on the enzyme derived from Pool II formed after Sephadex G-100 gel filtration. Column: 10 cm × 2 cm; hydrostatic pressure 150 cm; fraction size collected 2 ml; buffer 0.005 M β, β-dimethylglutaric acid pH 6.6, with an addition of a linear salt gradiation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

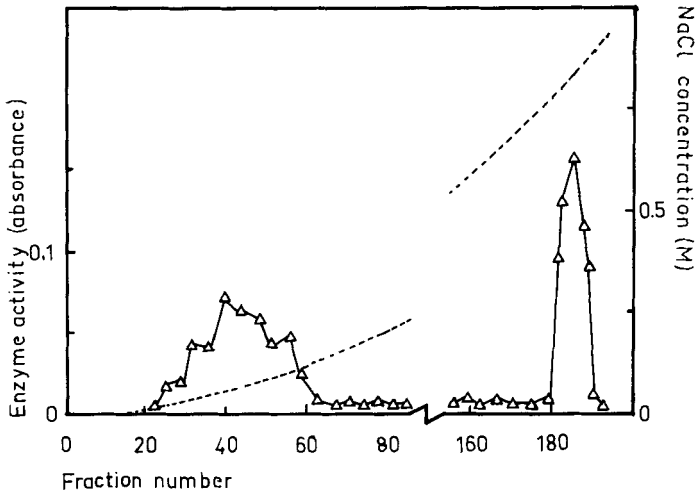

Fig. 7. DEAE-cellulose chromatography performed on the enzyme derived from Pool III formed after Sephadex G-100 gel filtration. Column: 10 cm × 2 cm; hydrostatic pressure 150 cm; fraction size collected 2 ml; buffer 0.005 M β, β-dimethylglutaric acid, pH 6.6, with an addition of a linear salt gradiation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

reaction mixture, and Mn^{2+} ions the weakest inhibitors of these enzymes. The other tested compounds showed no effect on the rate of hydrolysis of *N*-L-phenylalanyl-2-naphthylamine by any of the tested enzyme preparations.

DEAE-cellulose ionexchange chromatography on the pooled enzyme preparations. The enzyme preparations obtained after Sephadex G-100 gel filtration were transfered into 0.005 M β,β-dimethylglutaric acid by gel filtration through Sephadex G-25. Figures 5, 6, and 7 show the distribution of these enzymes capable of hydrolysing the working substrate after DEAE ionexchange chromatography. All enzymes of all pools were eluted off the columns by about the corresponding NaCl concentration values in the elution buffer. The ionexchange chromatography of these enzymes without prior transfering the enzyme into less concentrated elution buffer showed in all cases only one enzyme peak. Similar results were obtained when the enzymes were fractioned in presence of calcium in the elution buffer.

DISCUSSION

The chromatographic methods used in this study revealed a number of enzymes capable in hydrolysing the working substrate *N*-L-phenylalanyl-

2-naphthylamine. Sephadex G-100 gel filtration gave three different enzyme peaks, all of them having different Kd values, and having the same specific enzyme activity. This suggests of an existence of at least three different enzymes possessing this activity. The studies performed on the stability of the enzyme preparations towards pH changes showed, however, a rather similar behaviour, showing similar irreversible changes of the enzymes studied. These results suggest then that these enzymes could be the similar by some important properties. During the course of this study it could also be noted that the fractionated enzymes were extremely instable. Storage of the enzymes at +4°C resulted in a rapid loss of the tested enzyme activity from one day to an other supporting further the instability of the enzymes. The optimum pH was in all experiments around 7. These values should be interpreted with some precaution because a possible nonsaturation of the substrate in the enzymic reaction could exist at the different tested pH values. A nonsaturation of the substrate could result in a similar distribution of the experimental points in the Figures 3 A, B and C. The enzymes of Pool I were the only ones capable in hydrolysing the other tested substrate *N*-L-prolyl-2-naphthylamine. This enzyme activity showed a firm stability towards pH changes which was not the case with the experiments performed with the main working substrate. The pH dependence curve for the rate of hydrolysis of *N*-L-prolyl-2-naphthylamine is not ordinary bell shaped (Fig. 4) rather flattened off along the tested alkaline pH values. An enzymic behaviour like this suggests that the dissociation of enzyme substrate complex in the alkaline region evidently takes place through two differently protonated forms of the complex resulting in a higher rate of the hydrolysis at pH values above 8. A corresponding behaviour of the enzymes has already been reported by *Mäkinen* (1966, 1969), on enzymes derived from human whole saliva acting on *N*-L-prolyl-2-naphthylamine.

Of the metal cations only Ca^{2+} ions did activate the enzymes of Pool I and III, whereas the enzymes of Pool II were inhibited by these cation, suggesting that the enzymes of both Pool I and III could be metal dependent requiring Ca^{2+} ions in the enzymic hydrolysis reaction. EDTA, on the other hand, did inhibit the hydrolysis reaction of the enzymes of Pool II. This could be interpreted is such away that the inhibition of the enzymes of this Pool could not be based on a chelation of some important cations for the hydrolysis reaction, since both Ca^{2+} ions and EDTA ions produced the same effect on the enzyme reaction.

The wide range of the enzyme affectors were chosen for this study in terms of their known effect on proteolytic enzymes. DTT and L-cysteine usually activate so called SH-enzymes, NEM is known to inhibit the cor-

respoding enzymes, and TPCK reacts with α-chymotrypsine by histidine alkylation (*Schoellman & Shaw*, 1963). DPCC and PMSF also react with the active site of proteolytic enzymes, for example, chymotrypsine (*Erlanger et al.*, 1963 and *Gold*, 1965).

Of all the enzyme affectors selected for this study only L-cysteine did inhibit the enzymic reaction of the enzymes of Pool II and III leaving the enzymes of Pool I unaffected, suggesting that SH-groups would not be of primary importance in the hydrolysis reaction. L-cysteine probably reduces the disulphide bridges important for the maintenance of the structure of the active enzyme, followed by a loss of the important configuration.

All other affectors seemed to be inactive towards the enzymic hydrolysis reaction of *N*-L-phenylalanyl-2- naphthylamine.

A further fractionation of the enzymes after Sephadex G-100 gel filtration with DEAE-cellulose ionexchange chromatography showed that these enzymes could be fractioned in several components having this specific activity. However, the activity of the enzyme preparation were constantly to low to permit any closer investigation.

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SUMMARY

Human dental plaque was collected from different persons into 0.154 M NaCl solution and centrifuged. The enzymes dissolved in the saline solution were then fractioned by gel filtering through Sephadex G-100 according to their ability to hydrolyse *N*-L-phenylalanyl-2-naphthylamine. Three different enzyme pools (I, II, and III) were obtained having this specific activity. All the tested enzymes were extremely instable and they showed irreversible changes (reduction of activity) towards pH changes. Of the many different compounds and ions tested, only Ca^{2+} ions had an activating effect on the rate of hydrolysis of the substrate by the enzymes of Pool I and III. These enzymes behaved like metalloenzymes requiring Ca^{2+} ions in their hydrolysis reaction.

The results obtained in the experiments performed with L-cysteine revealed further that none of the tested enzymes would be typical SH-enzymes. Disulphide bridges are probably important in the maintenance of the structure of the active enzymes of Pool II.

A further DEAE-cellulose ionexchange chromatography revealed that the tested enzyme preparations could be fractionated into several components having this specific activity. However, no experiments could be performed on these enzymes, because of extremely low enzyme activities.

RÉSUMÉ

FRACTIONNEMENT DES ENZYMES DE LA PLAQUE BACTÉRIENNE DENTAIRE HUMAINE HYDROLYSANT LA *N*-L-PHÉNYLALANYL-2-NAPHLYLAMINE

Des prélèvements de plaque bactérienne dentaire humaine provenant de différentes personnes ont été recueillis dans une solution de NaCl 0, 154 M et centrifugés. Les enzymes dissous dans la solution saline ont ensuite été fractionnés par filtration sur gel sur Sephadex G-100 suivant leur aptitude à hydrolyser la *N*-L-phénylalanyl-2-naphtylamine. On a obtenu trois pools différents d'enzymes ayant cette activité spécifique. Tous les enzymes testés étaient extrêmement instables et présentaient des modifications irréversibles (reduction de l'activité) lors des changements de pH. Parmi les nombreux composés et ions testés, seuls les ions Ca^{2+} provoquaient une activation de la vitesse de l'hydrolyse du substrat par les enzymes du pool I et du pool III. Ces enzymes se comportaient comme des métallo-enzymes, les ions Ca^{2+} étant nécessaires à leur réaction d'hydrolyse. Les résultats obtenus dans les essais faits avec la L-cystéine ont de plus mis en lumière qu'aucun des enzymes testés ne serait typique du groupe SH. Les ponts disulfure sont probablement importants pour le maintien de la structure des enzymes actifs du pool. II.

La chromatographie par échange d'ions sur DEAE-cellulose a de plus montré que les préparations enzymatiques testées pouvaient être fractionnées en plusieurs constituants ayant cette acticvité spécifique. Cependant, il n'a pas été possible de faire d'expériences sur ces enzymes, en raison de leur activité enzymatique extrêmement faible.

ZUSAMMENFASSUNG

FRAKTIONIERUNG UND EINIGE EIGENSCHAFTEN VON ENZYMEN IN DER MENSCHLICHEN PLAQUE DIE N-L-PHENYLALANYL-2-NAPHTHYLAMINE HYDROLYSIEREN

Von verschiedenen Personen wurden Plaques in eine Lösung von 0.154 M NaCl gesammelt und zentrifugiert. Darauf wurden die in der Salzlösung befindlichen Enzyme durch Sephadex G-100 Filtration nach ihrer Fähigkeit *N*-L-Phenylalanyl-2-Naphthylamin zu hydrolysieren, fraktioniert. Man

erhielt drei verschiedene Enzymgruppen (I, II und III) mit dieser spezifischen Aktivität. Alle untersuchten Enzyme waren äusserst unstabil und zeigten irreversible Veränderungen (Aktivitätsverminderung) bei pH-Änderung. Von den vielen geprüften Verbindungen und Ionen hatte nur Ca^{2+} eine aktivierende Wirkung auf die Menge des Substrates, das durch die Enzyme der Gruppe I und II hydrolysiert wurde. Diese Enzyme verhielten sich wie Metalloenzyme, die Ca^{2+} Ionen für ihre Hydrolyse Reaktion benötigen.

Die Resultate der Experimente, die mit L-Cystein gemacht wurden erwiesen ferner, dass keines der geprüften Enzyme ein typisches SH-Enzym ist. Vermutlich sind Disulfidbrücken wichtig für die Erhaltung der Struktur der aktiven Enzymgruppe II.

Eine weitere DEAE-Ionenaustauschchromatographie zeigte, dass die getesteten Enzympräparate in verschiedene Komponenten, welche diese Eigenschaften haben, fraktioniert werden können. Mit diesen Enzymen konnten aber wegen ihrer niedrigen Aktivität keine Versuche angestellt werden.

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