From: The Institute of Dentistry, University of Turku, Finland

STUDIES ON ESTEROLYTIC ENZYMES FROM HUMAN PAROTID SALIVA

by

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INTRODUCTION

Since the litterature on the properties of salivary lipase contains many controversies (*Da-Rin,* 1930; *Katzenstein,* 1929; *Eggers-Lura,* 1946; *Scheer,* 1928; *Scheer & Fuhrberg,* 1928; *Vergin,* 1950), and since modern works of esterases are dealing with parotid gland homogenates and not saliva (*Arvy,* 1963; *Burstone,* 1956; *Hill & Bourne,* 1954; *Mahlers et al.,* 1955; *Rothers,* 1928) a more acurate study described below was undertaken. The presence of lipase in salivary glands has been claimed on the basis of histochemical studies, (*Martin,* 1953), an opinion not shared by *Abe et al.* (1964). Therefore a comparison between naphtylesters and natural fats as substrates was undertaken and the pH dependence of the activities and the effect of some known lipase inhibitors and activators mainly found in saliva were tested.

MATERIALS AND METHODS

Collection and preparation of saliva

The saliva was collected from the parotid duct with an acrylic cup modification of the Carlson-Crittenden device (*Shannon et al.,* 1962). The flow of saliva was stimulated with lime caramels (sweets) and collected from young healthy adult persons. The saliva was centrifuged immediately after the collection for 15 min at 40,000 $\times$ g. to remove all the adventitious particles. All

the procedures were performed under + 4° C conditions. The pooled saliva was concentrated 10 × by salting out with ammoniumsulphate to reach 100 % saturation. The precipitate was dissolved in distilled water. The concentrate was then dialysed against distilled water for 24 hours and then against 0,1 M tris-HCl buffer pH 7,0 with five changes until the Ba-test was negative.

Substrates

The following substrates were used: β-naphtyl-laurate, α-naphtyl-acetate, and triolein (Sigma Chemical Company, St. Louis, USA).

Affectors

The following affectors were used to characterize the enzymes: E-600, (diethyl-nitrophenyl phosphate, Mintacol, Bayer, Germany), cystein-HCl, sodium taurocholate, monojodo-acetic acid, EDTA disodium salt, and the following inorganic salts at final 4 mM concentration: $CuCl_2$, KCl, $HgCl_2$, $MgCl_2$, NaF, Na_2HPO_4.

Assays

Naphtol-substrates were dissolved in methanol to reach the strength of 1 mM. 0,1-M tris-HCl buffer was used. Mc-Ilvaine's phosphate, citric acid buffer was used when testing the pH optimum. The hydrolysis rate was measured in an incubation medium consistings of: 0,1-M tris-HCl or Mc-Ilvaine's buffer 1,0 ml, enzyme fraction 0,5 ml, substrate stock solution 0,5 ml. The incubation was carried out in a water bath at constant 37° C temperature from 6 to several hours, depending on the substrate used. When naphtyl substrates were used, the liberated naphtol was combined with diazonium salt by adding 0,5 ml of a 1 mg/ml containing aquous Fast Garnet GBC solution (Edward Gurr, Ltd. London, England). Two minutes later 1 ml 1-M acetate buffer pH 4,2 was added to stop the enzymic reaction. Color intensity was measured after 10 min at 530 mμ. The triolein substrate was prepared and the assays was performed as described by *Siegelmann* (1962), modifying the procedure to human saliva and triolein as follows:

0,2 ml of enzyme
0,8 ml of tris-HCl buffer
0,2 ml of substrate.

Incubation time was 44 hours. The reaction was stopped with 1 ml 0,6-M TCA and centrifuged for 20 minutes at 3200 rpm (Christ Junior III). After exactly 3 minutes was 0,1 ml $KMnO_4$ (2 %), 0,1 ml Na_2SO_3 (10 %) and chromotropic acid 4 ml added to 0,5 ml of the supernatant. The mixture was then stirred and boiled for 15 minutes and cooled for 3 minutes. The color extinction was measured at 580 mμ.

Column chromatography

DEAE-cellulose (Diethylaminoethylcellulose Whatman DE 11) was equilibrated with 20 mM tris-HCl buffer pH 7,0 and packed in a column of 2 $\times$ 43 cm. About 10 ml concentrated saliva was applied on the column. The best result was obtained with a continuous salt gradient from 0,5-M NaCl in 20-mM tris-HCl buffer pH 7,0. 5 ml fractions were collected and stored immediately in a refrigerator. CM-cellulose (Carboximethylcellulose Whatman D 70) was allowed to swell for two days in tris-HCl buffer pH 7,0, 0,1-m. The cellulose was then decanted 5 times with the same buffer and washed with 0,01-M NaOH containing 0,5-M NaCl. The equilibration was performed with 20-mM tris-HCl buffer pH 7,0. The cellulose was then packed in a column, as described earlier. 10 ml concentrated saliva was applied to the column, the procedure being the same as described above.

RESULTS

Studies on untreated saliva

The pH optimum of the hydrolysis rate of the stabilized duct parotid saliva was determined with β-naphtyl-laurate as the substrate. Mc-Ilvaine's phosphate-citric-acid buffer was used. The pH-curve (Fig. 1) shows that only one apparent activity maximum peak can be observed at the pH value 7. Sodium taurocholate, which is known to be a specific lipase activator, was used to test the occurence of lipase in stabilized untreated parotid saliva. The lipase activity was markedly increased.

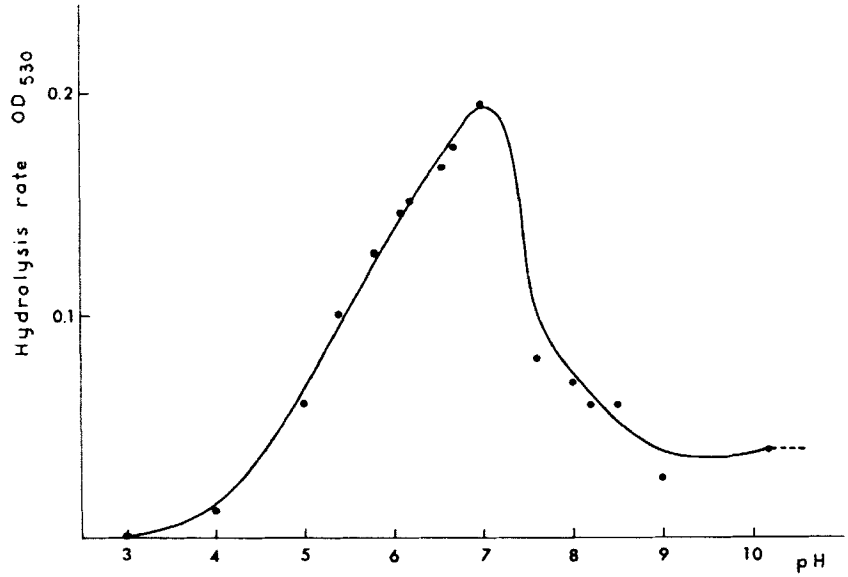

Fig. 1. The pH dependence of the hydrolysis rate of β-naphtyl-laurate as substrate. Untreated stabilized concentrated saliva in Michael's phosphate-citric acid buffer and tris-HCl buffer. Incubation time 24 hours.

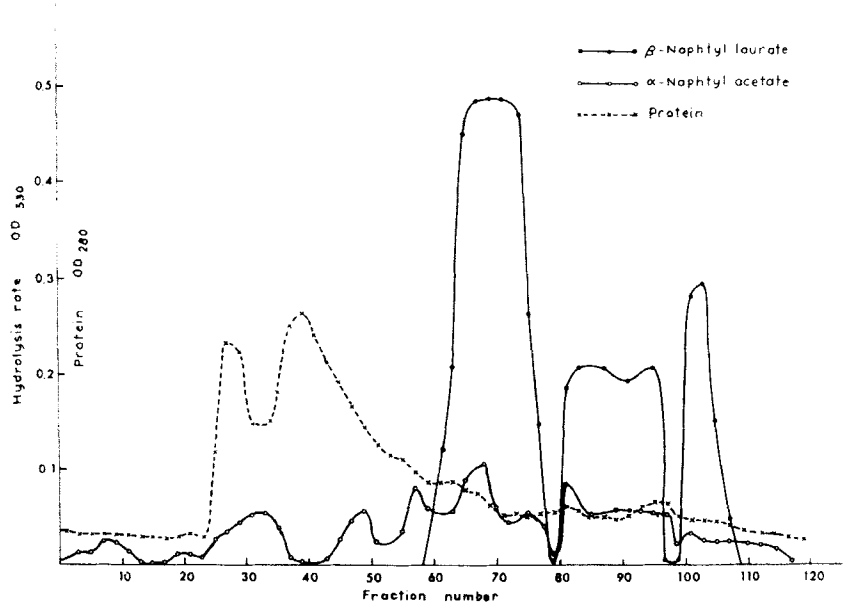

Fig. 2. Separation of enzymic activities hydrolysing β-naphtyl-laurate and α-naphtyl acetate using DEAE-cellulose chromatography 0,1-M-tris-HCl buffer pH 7. Incubation time 40 hours β-naphtyl-laurate and 12 for α-naphtyl acetate.

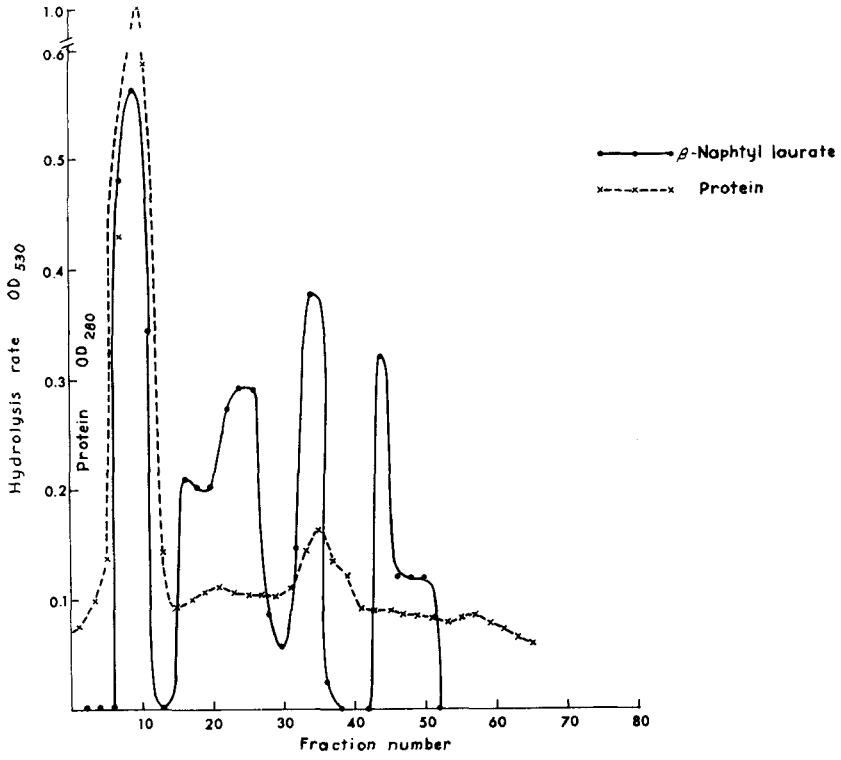

Fig. 3. Extinction values of the hydrolysis rate of β-naphtyl-laurate after CM-cellulose chromatography. Incubation time 40 hours.

The behaviour of the enzyme ion-exchange chromatography

All the runs were performed at pH 7,0 (Fig. 1). As can be seen from figure 2 and 3 with β-naphtyl-laurate, CM-cellulose compared with DEAE-cellulose gave better resolution with its apparent 5 enzyme peaks. The main portion of the protein in both methods was separated with the first fractions. (Fig. 2 and 3.)

Behaviour of the fractions

Hydrolysis of the ester- and lipase substrates was determined from the series of fractions of CM-cellulose chromatography. α-naphtyl acetate gave the greatest number of activities as demonstrated in Figure 4. β-naphtyl laurate gave five active peaks. The first peak of laurate around the fraction number 8 was identical

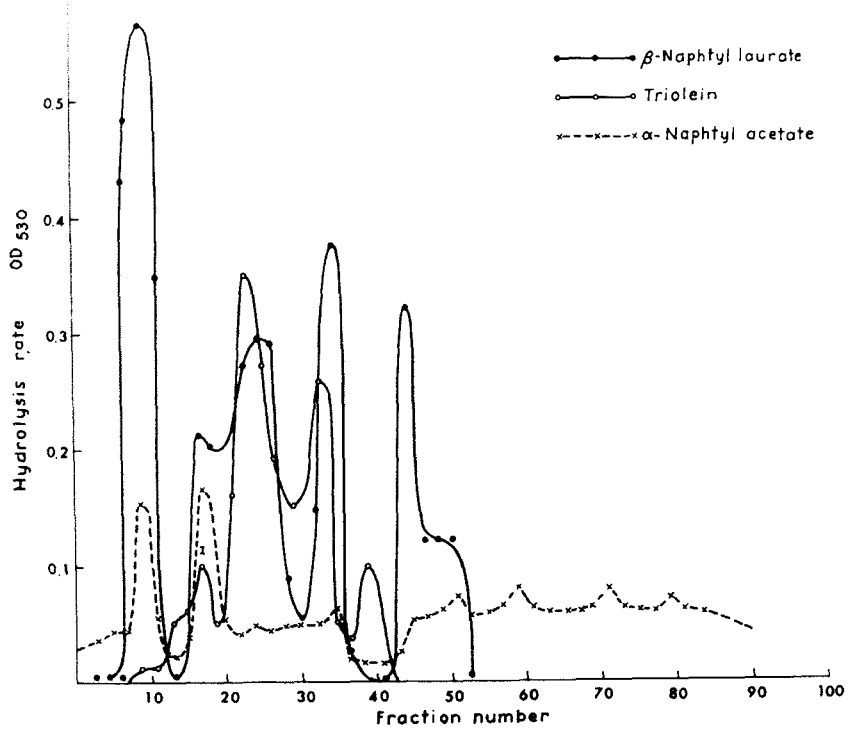

Fig. 4. CM-cellulose ion exchange. Separation of enzymic activities hydrolysing β-naphtyl-laurate, triolein and α-naphtyl acetate, 0,1-M-tris-HCl buffer, pH 7. Incubation time 40 hours for β-naphtyl-laurate, 48 hours for triolein and 12 for α-naphtyl acetate.

with the first acetate peak and the highest protein peak. Triolein as substrate gave four peaks of activity of which three were similar to those of laurate. (Fig. 4.)

Studies on pooled fractions

Two pools were made of the fractions number 11 to 29 and 30 to 40. These pools were further investigated. The pH depedence of the incubation medium on the hydrolysis rate was determined with β-naphtyl-laurate. The absolute heights of the activity peaks of the pools varied. Pool number 1 (11—29) appeared to be slightly lower than pool number 2 (30—40). Both pools showed the main activity peak to be around pH 7.

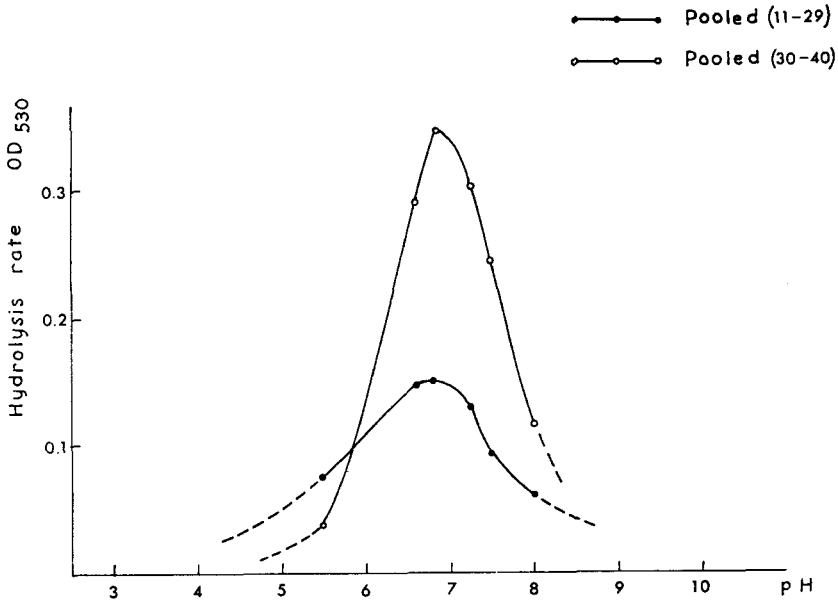

Fig. 5. The pH dependence of the hydrolysis rate of β-naphtyl-laurate as substrate after CM-cellulose chromatography.
Mc-Ilvaine's phosphate citric acid buffer was used. Incubation time 26 hours.

Table I. Effect of certain affectors on the hydrolysis of β-naphtyl laurate on pools 1 (11—29) and 2 (30—40) after CM-cellulose chromatography tested in 0,1-M-tris-HCl buffer pH 7,0.

Affector	Final conc. mM	Pool 1	Pool 2
E-600	0,02	0	0
$CaCl_2$	4	++	+++
$MnCl_2$	4	0	0
$CuCl_2$	4	———	———
$MgCl_2$	4	0	0
$HgCl_2$	4	——	——
EDTA	4	0	0
monojodacetate acid	4	0	0
cystein	4	0	0
KCl	4	0	0
Na_2HPO_4	4	0	0
Na-taurocholate	4	———	———
NaF	4	——	——

Studies with affectors

The effects of some affectors, known to modify the activity of the enzyme, were tested. Some of the affectors were chosen, because they are normally present in the oral environment, and because the effects are unknown. The results are given in Table I. It seems that the only slight activator of pool number 1 was $CaCl_2$, and pool 2 was activated more with $CaCl_2$; $CuCl_2$ and $HgCl_2$ slightly inhibited both pools, also. Sodium taurocholate inhibited the enzymes in both pools and all the fractions after CM-cellulose chromatography. The other affectors produced no notable effects on the enzymes.

DISCUSSION

At least five esterolytic enzymes in human parotid saliva were found after CM-cellulose chromatography. It was possible to demonstrate five different activity peaks with β-naphtyl-laurate as substrate and four with triolein as substrate. Three of the peaks were identical. On the basis of these results and as the lipase activators sodium taurocholate did increase the lipolytic activity in untreated stabilised parotid saliva, it can be expected that there are lipases in human parotid saliva. However, the tests with affectors showed that none of the fractioned enzymes were activated with sodium taurocholate, which is known to be a strong activator of lipase. On the contrary, sodium taurocholate did almost inhibit the activity of these enzymes. Because of the peculiar behaviour of the enzymes with sodium taurocholate, several explanations for the phenomena, can be given:

1) There is no typical lipase in human parotid saliva. 2) The lipase was inactivated during the chemical processes. 3) The lipase was so tightly bound to the ion exchange column that it was not possible to elute it off the column.

Our studies show that there could be several esterases in human parotid saliva, and that these enzymes can be separated in a rather pure form using ion exchange columns.

These apparent activity peaks could, however, be activities of only one enzyme, which for some reason behaves in this manner in the ionexchange column. It has also been suggested by *Hopsu & Glenner* (1963), that some proteolytic enzymes are capable of

hydrolyzing common chromogenic esters. *Riekkinen & Hopsu* (1965) showed that it was possible to separate twelve different esterolytic activities from rat submandibular salivary gland extract. Five of these activities appeared to hydrolyse preferentially trypsin substrates and only one of the activities showed typical characteristics of a protease. Further studies will reveal the real nature of these activities.

SUMMARY

Human parotid saliva was stabilized and concentrated. Both DEAE and CM-cellulose chromatography was performed. A better separation result was reached with CM-cellulose. We could separate at least five different activity peaks, which were able to hydrolyse β-naphtyl-laurate, and four activity peaks with the ability to hydrolyse triolein after a prolonged incubation period. The pH-optimum β-naphtyl-laurate as substrate was about 7. The fractioned enzymes were activated, among others, by $CaCl_2$ and inhibited by Na-taurocholate. It seems that no pancreatic type lipase is found in human parotid saliva, after ionexchange chromatography.

RÉSUMÉ

ETUDES SUR LES ENZYMES ESTÉROLYTIQUES DE LA SALIVE PAROTIDIENNE HUMAINE

De la salive parotidienne humaine a été stabilisée et concentrée. La chromatographie sur DEAE-cellulose et la chromatographie sur CM-cellulose ont été pratiquées. La CM-cellulose a donné la meilleure séparation. Nous avons pu séparer au moins cinq pics différents d'activité qui étaient capables d'hydrolyser le laurate de β-naphtyle, et quatre pics d'activité pouvant hydrolyser la trioléine après une période d'incubation prolongée. Le pH optimum du laurate de β-naphtyle comme substrat était environ 7. Les enzymes fractionnés ont été activés par $CaCl_2$ et inhibés par le taurocholate de Na. Il semble qu'il n'y ait pas de lipase du type pancréatique dans la salive parotidienne humaine, d'après la chromatographie sur échangeurs d'ions.

ZUSAMMENFASSUNG

UNTERSUCHUNG ÜBER DIE ESTEROLYTISCHEN ENZYME DES PAROTISSPEICHEL

Der Parotisspeichel von Menschen wurde stabilisiert und konzentriert. DEAE-Zellulose und CM-Zellulose Ionenaustauschkromatographie des Speichels wurde ausgeführt. Die Ionenaustauschkromatographie mit CM-Zellulose gab das bessere Resultat. Es gelang fünf Aktivitäten, die β-Naftyllaurat hydrolysieren und vier Aktivitäten, die Triolein hydrolysieren konnten, zu trennen. Das Optimale pH-Wert für die β-Naftyl-Laurat-Hydrolyse war pH 7. Die fraktionierten Enzyme wurden u.a. von $CaCl_2$ aktiviert und von Na-taurokolat inhibiert. Nach dem Ionenaustauschkromatographie-Verfahren lässt sich soweit im Parotisspeichel keine typische Lipase nachweisen.

REFERENCES

Abe, M., S. T. Kramer & A. M. Seligman, 1964: The histochemical demonstration of pancreatic-like lipase and comparison with distribution of esterase. J. Histochem. Cytochem. *12:* 364.

Arvy, L., 1963: Comparative histoenzymology of salivary gland cells. Ann. N. Y. Acad. Sci. *106:* 472.

Burstone, M. S., 1956: Esterases of salivary glands. J. Histochem. Cytochem. *4:* 130.

Da-Rin, O., 1930: Lipolytic Action of Saliva. Clin. med. ital. *61:* 340.

Eggers-Lura, H., 1946: A study of salivary lipase. Schweiz. Mschr. Zahnhk. *56:* 920 (Abstract. I.A.D.R. *34:* 652, 1947).

Hill, C. R. & G. H. Bourne, 1954: The histochemistry and cytology of salivary gland duct cells. Acta anat. (Basel) *20:* 116.

Hopsu, V. K. & G. G. Glenner, 1963: Further observations on histochemical esterase and amidase activities with similarities to trypsin. J. Histochem. Cytochem. *11:* 520.

Katzenstein, M., 1929: Studies of salivary lipase. Ztschr. ges. exper. Med. *69:* 179 (Biol. abst. 687, 1932).

Mahler, I. R., H. H. Chauncey & V. F. Lisanti, 1955: Lipolytic And Esterolytic Actixity of Salivary Organisms (Abstract.) J. Dent. Res. *34:* 709.

Martin, B. F., 1953: Lipase in gland duct epithelium and in mucus secreting cells. Nature *172:* 1048.

Riekkinen, P. J. & V. K. Hopsu, 1965: Studies on the salivary glands II. Hydrolysis of chromogenic ester substrates. Ann. Med. Exp. Fenn. *43:* 15.

Rother, P., 1928: Ueber Lipase im Speichel. Klin. Wschr. *7:* 163.

Scheer, K. & X. Fuhrberg, 1928: A salivary enzyme (lipase). Monatsch. Kinderheilk. *38:* Nos. 1—2, 115, (Biol. Abst. *4:* 21445, 1930.)

Shannon, I. L., J. R. Prigmore & H. H. Chauncey, 1962: Modified Carlson-Crittenden device for the collection of parotid fluid. J. Dent. Res. *41:* 778.

Siegelmann, A. M., A. S. Carson & T. Robertsohn, 1962: Investigation of serum trypsin and related substances. I. The quantitative demonstration of trypsin-like activity in human blood serum by a micromethod. Arch. Biochem. Biophys. *97:* 159.

Vergin, F., 1950: Biology of Saliva. Deutsch. dentist. Ztschr. *4:* 41.

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