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PARTIAL PURIFICATION AND CHARACTERIZATION OF ARYLAMIDASES FROM HUMAN PALATINE SECRETIONS

K. K. MÄKINEN,[1] EVA SÖDERLING,[2] K. K. VIRTANEN[3] and J. KOTIRANTA[3]

[1]School of Dentistry, The University of Michigan, Ann Arbor, MI 48109-1078, U.S.A., [2]Department of Biochemistry, Institute of Dentistry, University of Turku, Turku and [3]Department of Prosthetics, Institute of Dentistry, University of Oulu, Oulu, Finland

Summary—The secretions (HPS) contained an arylamidase-like enzyme discovered by chromatography on Sephadex G-100 Superfine columns using *N*-L-alanyl-2-naphthylamine (2NA) as substrate. The enzyme was fractionated in the void volume, suggesting that its molecular weight was 150,000 or higher. It hydrolysed, with decreasing rates, the 2NA of L-alanine, L-leucine, L-methionine and L-phenylalanine, the pH optimum for the best substrate (ala-2NA) being 8.0. α-Benzoyl-DL-arginine-2NA was not hydrolysed. *p*-Chloromercuribenzoate, EDTA, Ca^{2+} and Zn^{2+} were inhibitory, whereas chemical modification with typical tyrosyl group reagents did not significantly inactivate the enzyme. Treatment of HPS with Triton X-100 revealed two further arylamidase-like enzymes with lower mol. wt (90,000 and 40,000, respectively). Inhibition characteristics and Cl^- effects suggest that one of these enzymes resembles aminopeptidase *B* (EC 3.4.11.6). HPS also contains endopeptidase activity over a wide pH range (6–9). The number of enzymes in HPS is thus small and most of the peptidolytic activity of HPS *in vitro* is due to one major enzyme with arylamidase activity.

INTRODUCTION

We previously reported the presence in human palatine gland secretions (HPS) of arylamidases that hydrolyse *N*-L-alanyl-2-naphthylamine at a high rate (Mäkinen *et al.*, 1983, 1985). The overall specific arylamidase activity of HPS seemed to be higher than that of mixed saliva from the same subjects. We have now studied these arylamidase-like hydrolases in more detail.

MATERIALS AND METHODS

Collection and treatment of HPS

HPS was collected from 19 to 25 year old females using a filter-paper method (Mäkinen *et al.*, 1985). The samples were collected from subjects known to be good secretors of HPS. All collections were performed at 008–0010 h employing a standardized procedure of gentle mechanical stimulation of the palate with a round-ended instrument. The filter-paper discs were placed on each side of the soft and hard palates (Mäkinen *et al.*, 1985). After a 5-min collection, the paper discs were placed into test tubes containing 0.5 ml water (6 to 8 discs/tube) and stored at −20°C until sufficient HPS was obtained for enzyme purification. In a typical enzyme fractionation, 48–64 filter-paper discs from about 8 subjects were used. After 4-week storage at −20°C, 0.5 ml of 0.5 per cent Triton X-100 was added to each tube, their contents pooled and stirred with a magnetic stirrer for 90 min at 4°C, then centrifuged for 10 min at 20,000 *g* in the cold. The supernatant fluids were recovered and the filter-paper mass was again treated with 0.5 per cent Triton X-100 overnight, followed by centrifugation as above. The supernatant fluids were combined and concentrated to 1.0 ml with an Amicon YM 10 membrane. The concentrates were subjected to chromatography as below. In some experiments, the Triton treatment was replaced by a similar treatment with water only. The amount of HPS obtained from each subject was too small for chromatography. However, a few fractionations were performed with pooled samples of two subjects when the above ratio between the number of paper discs and the volume of the extraction solutions was maintained.

Chemical methods and chromatography

Arylamidase activity was determined using various *N*-L-aminoacyl-2-naphthylamines (2NA) as substrate (Mäkinen and Mäkinen, 1978). *N*-L-alanyl-2NA (ala-2NA) was hydrolysed most rapidly by the various HPS arylamidases and, accordingly, this substrate was chosen for various characterization studies. Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin as standard, and by absorption at 280 nm in chromatography. Chemical modification of HPS arylamidases was attempted using the procedures described by Mäkinen *et al.* (1982). Due to the small amounts of HPS obtained and the lability of the enzymes in several modifications, only diazotization and nitration of the arylamidases were successfully completed. These were performed with diazotized sulphanilic acid (DSA) and tetranitromethane (TNM) (Mäkinen *et al.*, 1982). DSA treatments were performed in 1.0-ml mixtures consisting of 0.2 ml of enzyme solution and 0.8 ml of 0.125 M Na_2CO_3 at 25°C. The pH of the mixtures was adjusted to 9.0 and the diazotization was initiated by adding a 25 μl portion of a freshly-prepared aqueous DSA solution (38 mg in 1 ml). The modification time was 10 min. Nitration of the enzymes with TNM was performed at 25°C in 1.0-ml reaction mixtures consisting of 0.2 ml of enzyme

solution, 0.8 ml of 20 mM tricine and 10 mM TNM. The reaction time was 60 min.

The substrate specificity of the enzymes was studied using various 2NA, casein (purified, Difco Laboratories, Detroit, Michigan, U.S.A.), denatured haemoglobin, a synthetic collagenase substrate (DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2; Serva, Heidelberg, FRG) and α-benzoyl-DL-arginine-2NA (BANA). The hydrolysis of casein and denatured haemoglobin was carried out in accord with Rick (1965). The cleavage of the DNP-peptide was determined as described by Masui *et al.* (1977) and that of BANA as described by Mäkinen and Mäkinen (1978). Thus, the endopeptidase activity was studied at 37°C and the 2NA derivatives of amino acids were tested at 30°C. Casein, haemoglobin and the DNP-peptide were tested with unfractionated HPS extracts only.

Descending molecular-permeation chromatography was performed on 143 × 1.6 cm Sephadex G-100 Superfine or Bio-Gel P-200 columns using 0.01 M ββ-dimethylglutarate buffer (pH 7.2) in equilibration and elution. The separations were carried out at 4°C using a constant flow rate of 50 ± 2 μl/min (adjusted with a pulsating pump). The fraction volumes were 1.0 ml. The active fractions were pooled and concentrated with an Amicon 4M 10 membrane to 1 or 2 ml.

RESULTS

Gel-permeation chromatography

Fractionation of the aqueous filter-paper extracts on Sephadex G-100 Superfine or Bio-Bel P-200 columns revealed one major enzyme with arylamidase activity. This enzyme (or group of closely-related enzymes) was eluted in the void volume. Assuming that this enzyme molecule is globular, its molecular weight can be estimated to be ≥ 150,000 (*vide infra*). The appearance of this enzyme in the void-volume fractions was a constant feature of all fractionations. Occasionally other peaks with arylamidase activity occurred in the eluates, but their activity was extremely low, or their appearance was rendered possible only by special treatment of the filter-paper discs. These treatments were: (1) Incubation of the filter-paper discs in Triton X-100 followed by centrifugation and single chromatography of the supernatant fluid on Sephadex G-100 Superfine gel. Rechromatography of the void-volume enzymes that did not contain Triton X-100, yielded one major enzyme. (2) Incubation of the paper discs in Triton X-100 followed by centrifugation and two consecutive gel filtrations, on Bio-Gel P-200 and Sephadex G-100 Superfine, respectively. The need to use a detergent in the separation of the low-molecular weight enzymes suggests that they occur *in vivo* as conjugated proteins. At this stage, all enzymes were nevertheless designated arylamidases (I, II and III). Figure 1 shows typical chromatograms which illustrate the separation of enzymes after special treatments of HPS (except Fig. 1B inset which represents a separation after extracting the paper discs with water). Most water extracts produced the void-volume enzyme arylamidase I only. Treatment of unused filter-paper discs with water or 0.5 per cent Triton X-100 showed that the paper extracts contained small amounts of protein, without proteolytic activity, which may have bound a certain amount of HPS peptidases in an unspecified way. However, the production by some HPS samples of only one enzyme peak in gel filtration (the void-volume peptidase), whereas some samples produced even three peaks, although similar filter-paper discs were used identically, suggests that non-specific binding of HPS peptidases to proteinaceous contaminants of filter paper was not significant.

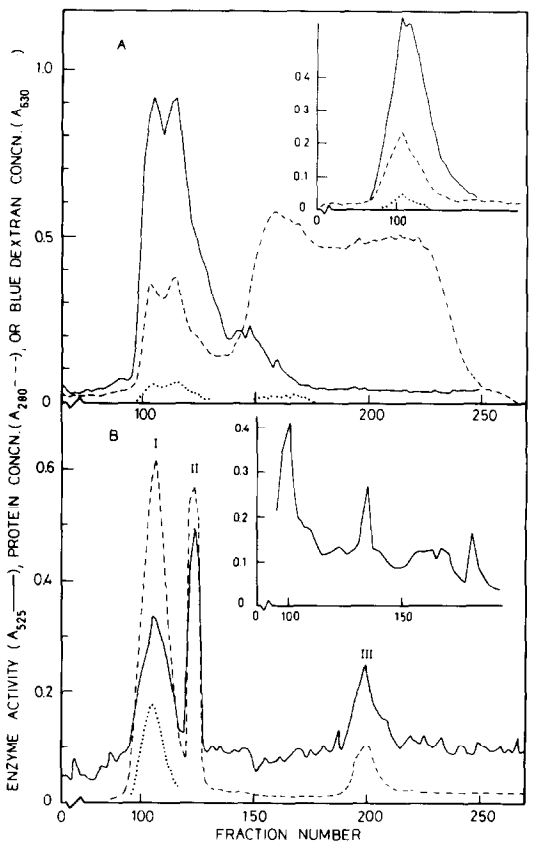

Fig. 1. Gel-permeation chromatography of HPS arylamidase-like enzymes. (A) Chromatography on a Sephadex G-100 Superfine column (1.6 × 143 cm) of HPS extracts obtained by treating the filter-paper discs from 8 subjects with Triton X-100. Elution buffer: 0.01 M ββ-dimethylglutarate buffer, pH 7.2. Sample: 1.5 ml of the Amicon concentrate of HPS extract plus 0.3 ml of 1 per cent blue dextran. Inset: Repetition of the chromatography with a sample obtained by concentrating fractions 95–130 from the first filtration. (B) Chromatography on a Sephadex G-100 Superfine column of a sample obtained by treating the filter-paper discs from 8 subjects with Triton X-100, followed by chromatography on Bio-Gel P-200 (no separation of arylamidases was achieved) and concentrating of the void volume fractions. Inset: Chromatography on a Sephadex G-100 Superfine column of an aqueous extract of filter-paper discs obtained from two subjects. Other details were as A.

The chromatograms obtained with the aqueous extracts of the filter-paper discs can be regarded as showing the normal state of HPS, the void-volume enzyme being a constitutive HPS hydrolase, and the smaller, occasional enzyme peaks standing for other

Table 1. Relative rate of the hydrolysis of *N*-L-amino-acyl-2-naphthylamines (2NA) by HPS arylamidases I, II and III, separated by molecular-permeation chromatography (Fig. 1B)

	Arylamidases		
Substrate	I	II	III
Ala-2NA	100	77	73
Arg-2NA	16	14	7
Gly-2NA	14	13	—*
Leu-2NA	46	57	52
Lys-2NA	3	—*	1
Met-2NA	48	33	—*
Phe-2NA	23	36	21
Pro-2NA	13	22	22

The rate of the hydrolysis of ala-2NA by enzyme I is marked as 100 and the others are given as relative values. The reactions were carried out in 0.025 M phosphate buffer, pH 7.2, without the addition of NaCl. The substrate concentrations were 0.167 mM.
*Not determined.

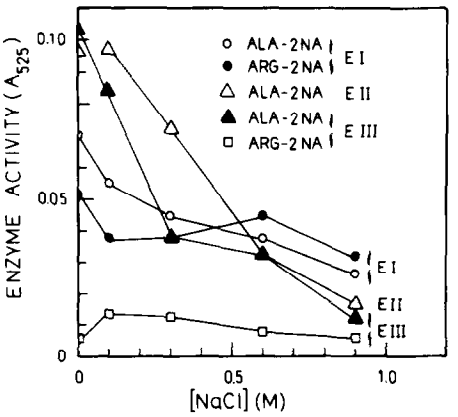

Fig. 3. Effect of NaCl on the rate of the hydrolysis of ala-2NA and arg-2NA catalysed by HPS arylamidases. The enzymes were obtained from gel-permeation chromatography of HPS (Fig. 1B). The reactions were performed in 0.01 M $\beta\beta$-dimethylglutarate buffer, pH 7.2, using 0.167 mM substrate concentration.

arylamidase-like enzymes with low activity. These occasionally-appearing low molecular-weight enzymes (Fig. 1B inset) did not coincide precisely with arylamidases II and III shown in Fig. 1B, probably because II and III may have been eluted as Triton-protein complexes. The activity of the smaller peaks in the inset of Fig. 1B was also too low for more detailed study. Therefore, the enzyme characterization below was carried out with those arylamidases (I, II and III) that were revealed after Triton treatment.

Substrate specificity

Ala-2NA and met-2NA were most rapidly hydrolysed by arylamidase I, whereas arylamidases II and III hydrolysed most rapidly ala-2NA and leu-2NA (Table 1). BANA, in which the α-amino function of arginine is blocked, was not hydrolysed by any of the enzymes even during prolonged incubation (18 h in 0.05 M tris–HCl buffer, pH 7.2 and 8.0).

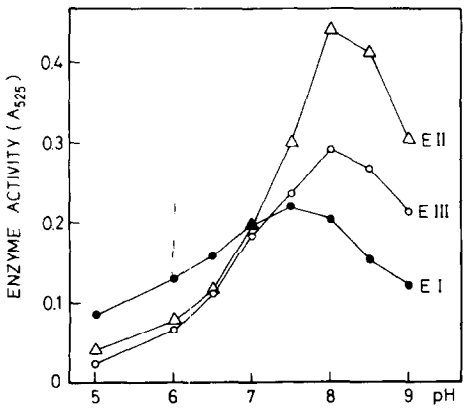

Fig. 2. Effect of pH of the rate of the hydrolysis of ala-2NA by HPS arylamidases. The reactions were performed in a mixture of 0.025 M phosphate buffer and 0.05 M tris–HCl buffer. The enzymes were obtained from gel-permeation chromatography of HPS (Fig. 1B).

The ability of the partially-purified arylamidases I–III to hydrolyse typical protease substrates, casein and haemoglobin, was not studied because the respective assay methods were not sufficiently sensitive. Therefore, the hydrolysis of these substrates was tested with unfractionated, aqueous filter-paper extracts only. A 17-h incubation with casein and haemoglobin as substrates revealed negligible enzyme activity only. The synthetic DNP peptide was hydrolysed at a clearly-measurable rate of 7.7×10^{-6} μmol/(min × mg; in a mixture of 0.025 M phosphate buffer and 0.05 M tris–HCl buffer).

Effect of pH

Enzymes I and II hydrolysed ala-2NA most rapidly at pH 8.0, whereas the pH optimum was at 7.5 for the high-molecular, dominant HPS arylamidase I (Fig. 2). With the DNP peptide, the pH optimum of the hydrolysis was at pH 7.0 (in a mixture of 0.025 M phosphate buffer and 0.05 M tris–HCl buffer).

Table 2. Effect of some reagents on the hydrolysis of *N*-L-alanyl-2-naphthylamine by arylamidases I, II and III in 0.025 M phosphate buffer, pH 7.2

	Arylaminopeptidase		
Reagent	I	II	III
5×10^{-7} M *p*CMB	25	0	0
1×10^{-6} M *p*CMB	28	10	10
1×10^{-5} M *p*CMB	93	100	100
5 mM EDTA	40	48	75
10 mM EDTA	46	57	84
1 mM $CaCl_2$	14	36	20
5 mM $CaCl_2$	42	50	40
5 mM $ZnCl_2$*	47	61	68
10 mM $ZnCl_2$*	78	66	90

The reactions were performed without added NaCl. The figures give the percentage of inhibition.
*Zn^{2+} precipitated a part of the enzyme. Absorbances were determined following centrifugation of the reaction mixtures.

Clearly-measurable activity was detected at pH 6.0, 8.0 and 9.0 as well. At pH 7.0, the hydrolysis of this substrate was inhibited by 50 per cent in the presence of 1.25 mM $CaCl_2$.

Effect of Cl^-

Figure 3 shows that the rate of the hydrolysis of ala-2NA catalysed by enzymes I and II was inhibited by NaCl. The rate of the hydrolysis of arg-2NA was either almost insensitive to the presence of NaCl (enzyme I), or the rate of the hydrolysis was strongly increased (enzyme III). No distinct maximum at 0.2 M NaCl, similar to that normally obtained for what can be called inflammatory arylamidases, was found. Therefore, the descending portion of the rate versus [NaCl] curves was not clearly bell-shaped either. Ther effect of 0.2 M NaCl, KCl, NaBr and LiCl on the hydrolysis of ala-2NA catalysed by enzyme I was also studied. The inhibitory effect of these salts decreased in the following order: NaBr < KCl ~ NaCl < LiCl.

Effect of some enzyme affectors

Table 2 shows that 10^{-5} M *p*-chloromercuribenzoate (*p*CMB) inhibited all enzymes strongly. EDTA also proved to be an inhibitor of these enzymes, enzyme III being inhibited slightly more than enzymes I and II. The enzyme activity appeared to be rather sensitive to the presence of Ca^{2+} and Zn^{2+}.

Chemical modification

Treatment of the high molecular-weight enzyme with DSA resulted in 32 per cent loss of enzyme activity. Modification with 10 mM TNM for 60 min did not affect the enzyme activity in any way. The lability of the enzymes under the modification conditions prevented more detailed experiments.

DISCUSSION

Our previous study indicated that HPS contains a small number of enzymes (Mäkinen *et al.*, 1983). The present study suggests that the number of HPS peptidases is also small, i.e. the overall arylamidase activity of HPS is caused by one major, high-molecular enzyme which does not hydrolyse BANA. The gel columns used in the fractionations had an effective separation capacity; in the molecular-weight range involved, they functioned linearly and separated globular proteins with an accuracy of ±10,000 (Virtanen, Mäkinen and Oksala, 1977; Mäkinen and Mäkinen, 1978). This major enzyme (or group of closely-related enzymes) appeared to be a high-molecular hydrolase because it was constantly eluted in the void volume and it was present *in vivo* in all HPS samples studied. The arylamidase-like enzymes II and III occurred in small amounts only. Their slightly varying inhibition and specificity characteristics do not entitle us to regard them as distinctly-separate arylamidases and they do not necessarily correspond to the small enzyme peaks shown in Fig. 1B (inset) either.

The HPS arylamidases were labile; after desorption of the enzymes from the filter-paper discs, the activity was gradually lost, especially after chromatography; this did not allow us to carry out a thorough enzyme purification and characterization that have normally been perfomed with certain other arylamidases (Mäkinen and Mäkinen, 1978). Storage of intact HPS samples at −20°C did not affect the arylamidase activity particularly. It is possible that the use of filter paper in the collection of HPS improved the purification of the enzymes because the paper absorbs considerable amounts of glycoproteins that are difficult to extract thoroughly with Triton X-100 or water (Mäkinen *et al.*, 1985).

The designation of these enzymes as arylamidases stems from the use of amino acyl-2NA as working substrates. Provided the hydrolysis of the amino acyl-2NA and the DNP-peptide was caused by one and the same high-molecular enzyme, at least arylamidase I should more correctly be termed an unspecific endopeptidase with arylamidase activity rather than a true arylamidase. The present data does not entitle us to classify them into any of the known mammalian proteinases. It may be speculated, however, that the inability of the enzymes to hydrolyse BANA does not relate them to cathepsin *B*, for example. The DNP-heptapeptide has been used as a substrate of mammalian collagenases (Masui *et al.*, 1977). The cleavage of this substrate by HPS does not indicate that HPS would contain a true collagenase. Our conclusion is that this hydrolysis was due rather to the action of a peptidase (enzyme I) other than a true collagenase. The hydrolysis of the DNP-peptide by HPS may be regarded as an important finding, as it suggests that this secretion contains a specific, novel endopeptidase.

The sensitivity of the enzymes to *p*CMB (and Zn^{2+}) suggests that they resemble sulphydryl proteinases. On the other hand, inhibition by EDTA does not indicate that these enzymes are metalloproteases; EDTA may act unspecifically as well and not as a chelator. Chelation is unlikely because Ca^{2+} inhibited these enzymes. Treatment of enzyme I with TNM and DSA under conditions that have led to a prompt nitration or diazotation of several enzyme proteins (Mäkinen *et al.*, 1982) had only a marginal effect. This suggests that tyrosyl residues were not essential for the activity of the high-molecular enzyme.

The hydrolysis of ala-2NA was sensitive to inorganic salts in general and Ca^{2+} and the alkali metal halides proved to be inhibitory. The hydrolysis of arg-2NA was not as sensitive to inorganic salts. It is thus likely that these enzymes display different sensitivity to salts depending on the nature of the substrate. Similar results were obtained with erythrocyte arylamidases (Mäkinen and Mäkinen, 1978).

The behaviour with Cl^- resembled that of most other mammalian arylamidase-like enzymes hydrolysing ala-2NA and arg-2NA; Cl^- inhibited the hydrolysis of ala-2NA and enhanced that of arg-2NA. The arylamidase activity of porcine dental pulp (Mäkinen, Brummer and Scheinin, 1970) and human erythrocytes (Mäkinen and Mäkinen, 1978) also behaved in this way. The varying degrees of activation of HPS arylamidases by Cl^-, observed with arg-2NA as substrate (Mäkinen *et al.*, 1983, 1985), may be explained as variations in the activity or concentration of enzyme III under different conditions. However, these data did not convincingly demonstrate in HPS the presence of a true aminopeptidase

B (EC 3.4.11.6), which is a typical arginine aminopeptidase of inflammatory exudates and many mammalian tissues (Mäkinen and Mäkinen, 1978). Evidence that would support the involvement of this type of enzyme in HPS is the shape of the rate versus [NaCl] curve for arg-2NA catalysed by enzyme III (Fig. 3), which resembles the curvature occasionally obtained with certain aminopeptidase *B*-like enzymes (Virtanen *et al.*, 1977), although complete bell-shape was not observed; bell-shape is a characteristic of the Cl^--dependent inflammatory arylamidases. Further evidence would be that both enzyme III and aminopeptidase *B* were inhibited by *p*CMB and EDTA (Virtanen *et al.*, 1977; Mäkinen and Mäkinen, 1978). The arg-2NA hydrolysing aminopeptidases from human palatinal mucosa showed a similar dependence on Cl^- as the present enzymes (Virtanen *et al.*, 1977).

It is difficult to suggest a biological role for the HPS enzymes. Their specificity suggests, however, that they are able to cleave complex protein molecules. At least one of the HPS enzymes (or a molecular form of enzyme I) has properties that resemble those of the so-called inflammatory aminopeptidase *B*. Because all the HPS samples contained at least the high-molecular enzyme I, it is likely that its presence in HPS is important to the normal functions of this secretion. It is also possible that some of the arylamidase-like enzymes, especially those with low activity (Fig. 1B), represent peptidases involved in the synthesis and modification of the major glycoproteins of HPS. Such enzymes would thus have little significance in the mouth. Therefore, future studies need to compare the low molecular-weight enzymes with the signal peptidases of the secretory cycle. Based on our substrate-specificity studies, it appears that the HPS peptidases are able to hydrolyse similar substrates to those of the enzymes present in saliva from the major salivary glands. The high molecular-weight enzyme in HPS can be considered to contribute more significantly to the overall proteolytic capacity of oral fluid.

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