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The Proline Iminopeptidases of the Human Oral Cavity

Partial Purification and Characterization

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Enzymes possessing proline iminopeptidase activity were studied with various enzyme preparations from human oral cavity and certain micro-organisms using the 2-naphthylamides of L-proline and L-hydroxyproline as main substrates. The richest source of this enzyme activity in the human oral cavity was the bacterial plaque on the teeth, the bacterial covering of other oral surfaces, and carious dentine. Most of the salivary proline iminopeptidase activity was found to originate in the oral micro-organisms. Fractionation of bacterial plaque (treated with ultrasonic disintegrator) revealed three enzymes (I, II, and III) possessing proline iminopeptidase activity. One of these (III) was more thoroughly purified from human whole saliva. One of the enzymes (I) was found to be unspecific as regards the amino acid portion of the substrate, the 2-naphthylamides of L-alanine, L-methionine, L-leucine, and L-phenylalanine being hydrolyzed most rapidly. II and III were considered true proline iminopeptidases: only the 2-naphthylamides of L-proline and L-hydroxyproline were hydrolyzed at a significant rate.

The more thoroughly purified enzyme (III) was tested with certain peptides: L-prolyl-L-phenylalanine and L-prolylglycine were hydrolyzed. No action on poly-L-proline, poly-L-hydroxyproline, and poly-*O*-acetyl-L-hydroxyproline was detected. The value of the apparent Michaelis constant with L-prolyl-2-naphthylamide was 1.0×10^{-3} M, 1.0×10^{-4} M, and 1.0×10^{-4} M, for I, II, and III, respectively. The graphical methods of plotting revealed substrate inhibition, particularly with II and III. *N*-Ethylmaleimide and *p*-chloromercuribenzoate inhibited the action of I, but not that of II or III. The enzymes were not metal dependent. The K_i values obtained with diphenylcarbamyl chloride were: 2.8×10^{-4} M, 3.2×10^{-4} M, and 1.5×10^{-4} M, with the three enzymes, respectively, indicating certain similarities to the action of trypsin. Other experiments did not reveal the importance of an imidazole as a nucleophile in the action of any of the enzymes, nor did they reveal the participation of an active serine residue.

The ability of several cultivated micro-organisms to synthetize enzymes possessing proline iminopeptidase activity was also studied. In the conditions employed, the cells of *Lactobacillus casei* were found to be effective formers of enzymes displaying iminopeptidase activity, whereas those of *Str. salivarius*, for example, were not. The formation of proline iminopeptidase-like enzymes in *L. casei* cells started almost

simultaneously with the cell division and continued during the whole growth cycle. A small portion of this enzyme activity was encountered in the growth medium.

The significance of the plaque and salivary enzymes as regards oral biochemistry is discussed and certain properties of various iminopeptidases and arylaminopeptidases described in the literature are briefly compared with those of the three proline iminopeptidases discovered.

The only proline iminopeptidase (L-prolyl-peptide hydrolase, EC 3.4.1.4) thus far studied more thoroughly is that of a prolineless mutant of *Escherichia coli* [1] although an iminodipeptidase (L-prolyl-amino-acid hydrolase, prolinase, EC 3.4.3.6) is known to hydrolyze a variety of dipeptides also containing *N*-terminal proline.[2,3] Recently, the occurrence of proline iminopeptidase activity has been briefly studied in human whole saliva, in the bacterial covering (bacterial plaque) of the teeth and other oral surfaces, and in guinea pig saliva, using L-prolyl-2-naphthylamide as substrate.[4–6] It had been found that human whole saliva and bacterial plaque contain enzymes possessing considerable proline iminopeptidase activity. Such enzyme activity was almost never found in the saliva of guinea pigs (and dogs according to more recent studies in this laboratory), or else it was present in very low quantities.[6] On the other hand, these animals do not normally suffer from dental caries, or at least it is not very common among them, nor do they normally suffer from those inflammatory diseases of gingiva (periodontal diseases) which are very common in man. These observations led to a study of the enzymatic differences in the salivas of human and animal species in order to determine the possible role of enzymes in oral diseases, as well as their contribution to oral biochemistry. It was reasonable to assume that bacterial enzymes, like the proline iminopeptidases studied in this paper, contribute to the degradation, for example, of the connective tissue in the periodontal ligament which fastens the teeth to the bone. This idea has had some support from studies concerning the action of a crude bacterial plaque enzyme preparation on insoluble collagen isolated from human periodontal ligament.[7]

This paper will provide information about the existence and the chemical characteristics of enzymes in the human oral cavity capable of hydrolyzing L-prolyl-2-naphthylamide. These enzymes may also be called arylimino-peptidases, much in accordance with the term arylaminopeptidases (cleaving amino acid 2-naphthylamides) suggested, among others, by Nachlas *et al.*,[8] for an enzyme cleaving L-leucyl-2-naphthylamide. One of the goals of the present study has been to demonstrate that most of the proline iminopeptidases found in the oral cavity is formed in oral micro-organisms and then liberated from them into their environment.

For enzymes acting on 2-naphthylamides of various amino acids with free α-amino group, a somewhat similar mechanism of action can be suggested. There is, however, not very much information about the processes, or the characteristics of the active sites, of arylaminopeptidases. One of the more familiar arylaminopeptidase-like enzymes, in this sense, is aminopeptidase B,

specifically cleaving the 2-naphthylamides of L-arginine and L-lysine.[9,10] Kinetic and other experiments[11–13] on aminopeptidase B have suggested the following amino acid side groups as important for the action of the enzyme: the imidazole group of a histidine residue, the SH-group of a cysteine residue, and the α-amino group of the substrate. Because there was no similar report on the action of enzymes possessing aryliminopeptidase activity, this paper will, therefore, also offer information on some effects of pH and certain chemical compounds on the action of the enzyme involved.

MATERIAL AND METHODS

1. Enzymes. Leucine aminopeptidase, grade III, (L-leucyl-peptide hydrolase, EC 3.4.1.1) from hog intestine, subtilisin, grade VIII, (subtilopeptidase A, EC 3.4.4.16) from *Bacillus subtilis*, and chymotrypsin A, type II, (EC 3.4.4.5) from bovine pancreas were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Trypsin (EC 3.4.4.4) was purchased from Novo Industri A/S (Copenhagen, Denmark) by the trade name Trypure®, and papain (Verdauungsvermögen 1:350) (EC 3.4.4.10) was a product of E. Merck AG (Darmstadt, Germany). The dry enzyme preparations were stored in the cold according to the instructions of the manufacturers. The enzymes were dissolved in cold buffer immediately before use.

2. Other chemicals. 2-Naphthylamides of amino acids were purchased from Mann Research Laboratories, Inc. (New York, N.Y., USA) as well as the following compounds: *N*-ethylmaleimide, phenylmethanesulphonyl fluoride, diphenylcarbamyl chloride, acetylmercaptosuccinic anhydride, *p*-toluenesulphonyl-L-phenylalanylchloromethyl ketone, and glycyl-L-hydroxyproline, glycyl-L-prolylglycylglycine, L-hydroxyprolylglycine, L-prolyl-L-phenylalanineamide HCl, L-prolylglycine hydrate, poly-L-proline, poly-L-hydroxyproline, and poly-*O*-acetyl-L-hydroxyproline. Glycyl-L-proline was purchased from Sigma Chemical Co. Dithiothreitol and *N*α-tosyl-L-lysyl chloromethane were products of Calbiochem (Los Angeles, Calif., USA). Diazotized 4-amino-3,1′-dimethylazobenzene (Fast Garnet GBC Salt) was a product of G.T. Gurr (London, England). Most of the ingredients of the growth mediums of micro-organisms were purchased from Difco Laboratories (Detroit, Mich., USA). Unless otherwise stated all other chemicals were purchased from E. Merck AG.

3. Enzyme preparations from the oral cavity. In this investigation the proline iminopeptidase activity of the following samples obtained from human oral cavity was studied: whole saliva, parotid saliva, bacterial plaque, carious dentine, and normal dentine. Bacterial plaque refers to that soft white or yellowish material which was easily removed from the surface of the teeth when collecting the whole saliva by paraffin stimulation. The material remained as a pellet at the bottom of the centrifuge tube when the whole saliva sample was centrifuged. The material obtained in this way was termed Bacterial Plaque I. Bacterial plaque (Bacterial Plaque II) was also collected directly from the surface of the teeth with an excavator. This material may have had a composition differing from that obtained from centrifuged whole saliva because the latter material also contained micro-organisms from other oral surfaces, as a result of the rubbing action of paraffin chewing. In each case, however, both of the plaque preparations may be said to have been composed of micro-organisms, mucins, polysaccharides, *etc.*[14]

Carious dentine means that material obtained from extracted teeth with advanced caries. The carious lesions were somewhat demineralized and pigmented, and they were found to contain large numbers of various micro-organisms. All of the aqueous enzyme preparations obtained from these materials were stored, frozen at −20°C, between experiments. The enzyme preparations were diluted in a proper ratio with cold water just before use. The exact manner of collecting and handling the oral enzyme preparations follows, in principle, the methods used earlier.[15,26] In addition, the ability of some commercial proteolytic enzyme preparations to hydrolyze L-prolyl-2-naphthylamide was also studied for comparisons. These enzymes were leucine aminopeptidase, subtilisin, trypsin, chymotrypsin, and papain.

Table 1. List of micro-organisms used in the study and some details on their cultivation. The first column gives the names of the test organisms, the second column gives the length of the incubation time in the final cultivation in 500 ml of TSHGA medium. The other columns give further information on the cultivations of the test organisms.

Test organism	Incubation time (h)	Mg of cells (dry weight) in 1 ml of growth medium at the end of cultivation	Turbidity reading reached	Final pH of the growth medium[d]
Lactobacillus casei NCTC 10302[a]	8.7	0.18	70	5.3
Lactobacillus casei ssp. *rhamnosus* ATCC 7469[b]	7.8	0.13	45	5.3
Lactobacillus acidophilus NCTC 1899[a]	8.7	0.20	77	5.3
Lactobacillus fermenti ATCC 9338[a]	9.0	0.20	82	5.2
Lactobacillus lactis ATCC 8000[b]	4.5	0.19	80	4.5
Streptococcus sp. ATCC 9854[b]	4.5	0.24	103	4.9
Streptococcus pyogenes ATCC 6636[b]	4.0	0.18	75	4.5
Streptococcus pyogenes (*haemolyticus*) ATCC 9342[b]	6.5	0.20	87	4.3
Streptococcus salivarius[c]	24	0.24	104	4.4
Streptococcus sp. *Viridans* type NCTC 3165[a]	6.7	0.12	48	4.9
Corynebacterium bovis NCTC 3224[a]	6.5	0.35	140	5.5
E. coli 113[b]	3.0	0.35	129	5.5
E. coli 154[b]	5.0	0.21	83	5.1

[a] Provided originally by National Collection of Type Cultures, NCTC (Central Public Health Laboratory, London, England).

[b] Provided originally by Apteekkitavaraintarkastuslaboratorio Oy, Helsinki, Finland. The numbers refer either to the American Type Culture Collection numbers (ATCC), or, in the case of the two Escherichias, to numbers given by the supplier.

[c] Isolated at this laboratory from human whole saliva.

[d] Measured at 37°C with glass electrodes just before harvesting the cells.

4. Enzyme preparations from cultivated micro-organisms. For this investigation 13 different micro-organism species or strains were chosen. A number of them are closely related to those constantly occurring in the human mouth. The goal was to elucidate the ability of various micro-organisms in general to form enzymes capable of hydrolyzing L-prolyl-2-naphthylamide. The test organisms are listed in Table 1 together with certain information on their source and cultivation. The final cultivations were performed under identical conditions so as to achieve reliably greater comparability between the results. The exact methods used in cultivations and in making the bacterial enzyme preparations are given elsewhere.[15]

When the biosynthesis of proline iminopeptidase in the cells of two bacteria closely related to those constantly occurring in the human oral cavity (*L. casei* and *Str. salivarius*) was studied, 10 ml samples were taken at proper time intervals from the 500 ml TSHGA-medium. The cells from these samples were harvested by centrifugation and handled in the same way as presented elsewhere.[15] The 10 ml supernatant fluids were saved. The enzyme preparations from the cells and the medium were stored at −20°C until used (about 15 h).

5. Dry weight of micro-organisms. The dry weight of the micro-organisms studied was determined from one 10 ml sample from the same growth phase from which the cells had been harvested for enzyme studies. The drying was conducted with centrifuged pellets [at 23 500 *g* (14 000 rpm) at 4°C for 10 min; washed thrice with cold 0.9 % NaCl]

at 65°C until a constant weight was reached. For most test organisms a colorimeter reading of 100 (with the Klett-Summerson colorimeter; filter No. 62) corresponded to 0.2—0.3 mg dried cells per ml growth medium.

6. Column chromatography. The gel filtrations were conducted on Sephadex® columns. In the preparation of gels and in conducting the fractionations, the instructions of the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden) were followed. DEAE-cellulose chromatography was carried out on columns packed with a product of Schleicher & Schüll (Dassel/Kr. Einbeck, Germany). A fraction of 200—230 mesh was sieved from the commercial preparation. In preparing the material, in packing the columns, and in conducting the fractionations, the instructions presented by Peterson and Sober have been followed.[16]

7. Measurement of sodium chloride gradients. The exact form of the sodium chloride gradients in the DEAE-cellulose chromatography was determined by measuring the sodium content of the fractions with the Perkin Elmer Atomic Absorption Spectrophotometer Model 303, at 3302 Å in the UV-range. Aliquots of 25 μl from the fractions were diluted with varying amounts of water (1—6 ml), depending on the concentration of sodium. The results showed that the form of the gradients was not strictly linear.

8. Determination of protein concentration. In this investigation the protein concentrations were determined using the Folin-Ciocalteu method.[17] Bovine serum albumin (crystallized and lyophilized, Sigma Chem. Co.) was used as a standard.

9. Determination of enzyme activity. The estimation of proline iminopeptidase activity was performed in a mixture containing the following ingredients: 0.3 ml of buffer, 0.1 ml of 10^{-3} M aqueous L-prolyl-2-naphthylamide solution, 0.1 ml of water (which could be replaced by solutions of various enzyme inhibitors or other compounds), and 0.1 ml of enzyme preparation. The mixtures were incubated for various periods of time at 37°C and the enzyme reactions were stopped by adding 0.2 ml of 0.1 % Fast Garnet Salt GBC (diazotized 4-amino-3,1′-dimethylazobenzene) in 1 M acetic buffer, pH 4.2, which contained 10 % Tween-20 (v/v). The colour intensity was read on a Hitachi Perkin Elmer 139 Spectro-photometer at 525 mμ. A standard curve was prepared with free 2-naphthylamine (Sigma Chem. Co.). Usually, the cold solutions were pipetted into cold test tubes in an ice bath (about 4°C) after which the tubes were put in the 37°C water bath. More detailed information about the buffers, *etc.*, is given with each experiment later.

The amino acid 2-naphthylamides were, when possible, dissolved in water. The water soluble substrates were especially the 2-naphthylamides of L-arginine, L-isoleucine, L-leucine, L-lysine, and L-proline. Most of the other substrates were dissolved well in 5 or 10 ml ethanol and were not precipitated when made up to 100 ml with water. The most insoluble substrates were used as homogeneous aqueous suspensions. If the compound in question did not form any homogeneous suspension, the mixtures were treated with an Ultra-Turrax top drive homogenizer (Janke & Kunkel, Stauffen i.Br. Germany) for 60 sec at room temperature, to reduce the size of the undissolved particles. When these suspensions were used as substrates in enzyme reactions, the reaction mixtures also became slightly turbid and were saturated by the substrate involved during the whole reaction. However, when the reactions were stopped by the acetic acid-tween-diazonium salt mixture, the insoluble substrate particles were all dissolved, and the readings on the spectrophotometer could be performed with clear solutions.

The ability of the enzyme preparations studied to hydrolyze 2-naphthylamides of other amino acids was studied in the above manner, but replacing the L-prolyl-2-naphthylamide solutions with solutions of the other substrates. The eventual turbidity in the reaction mixtures due to the use of concentrated enzyme preparations was removed, after adding the diazonium salt, by centrifuging at 4500 rpm (3100 *g*) for 10 min at room temperature (with the Laboratory Centrifuge Universal Junior III).

The ability of commercial preparations of leucine aminopeptidase, subtilisin, trypsin, chymotrypsin, and papain to hydrolyze L-prolyl-2-naphthylamide was studied in principle in the same way as the proline iminopeptidases. On the basis of some preliminary experiments, these enzymes were studied in the following concentrations (μg in the reaction mixture of 0.6 ml): Leucine aminopeptidase: 25 μg; subtilisin: 300 μg; chymotrypsin: 200 μg; trypsin: 5 μg; papain: 100 μg. More detailed information about the conditions is given with each experiment later.

Because the presence of reducing compounds at higher concentrations in the reaction mixture interfered with the coupling reaction between 2-naphthylamine and diazotized

4-amino-3,1′-dimethylazobenzene, all of the experiments, with protein modifying compounds possessing reducing groups or compounds otherwise interfering with the determinations, were carried out by stopping the enzyme reactions using the method of Bratton and Marshall.[18] Such compounds were L-cysteine, dithiothreitol, and phenylmethanesulphonyl fluoride. In addition, the other compounds used to investigate the inhibition and activation of the enzymes interfered at higher concentrations, but these effects were controlled by proper blanks. *N*-Ethylmaleimide, *p*-toluenesulphonyl-L-phenylalanyl chloro-methyl ketone, *N*α-tosyl-L-lysyl chloromethane, dithiothreitol, L-cysteine, and EDTA were dissolved in water. EDTA formed turbid suspensions at higher concentrations. The particles were, however, dissolved when the homogeneously shaken suspension was added to the reaction mixture. Diphenylcarbamyl chloride solutions were prepared daily in acetone. The other compounds used were dissolved in 5 ml of ethanol and made up to 100 ml with water. The various dilutions from these solutions were made using 5 % ethanol (v/v). When the influence of these compounds on the enzyme reactions was studied, special blanks were also prepared in which the effect of the above mentioned organic solvents was studied. Solutions made from all other compounds except for those of diphenylcarbamyl chloride and *N*-ethylmaleimide, were found to be rather stable when stored at 4°C during the study.

The hydrolysis of di- and tetrapeptides was studied in reaction mixtures containing the following ingredients: 1) 50 μl of 0.05 M phosphate buffer, pH 7.0, in which the peptides were dissolved to a concentration of 0.5×10^{-3} M; 2) 50 μl of enzyme preparation. The mixtures were incubated for 3 h at 37°C and the reactions were stopped by placing the test tubes in a boiling water bath for 5 min. Mixtures in which the enzyme or substrate was replaced with water were also included. Because no turbidity was formed when stopping the reactions, the solutions were not centrifuged. Aliquots of 20 μl were applied on Silica Gel G thin layer plates and analyzed as described in the following section. When poly-L-proline, poly-L-hydroxyproline, and poly-*O*-acetyl-L-hydroxyproline were used as substrates, 2 mg of the dry substances was first weighed in the bottom of small test tubes, followed by 0.1 ml of the above buffer. After 24 h, standing at room temperature, the first two mentioned substrates were dissolved, while the third compound remained undissolved. 0.1 ml of the same enzyme preparation was added and the mixtures were incubated for 3 h. The tubes were then treated and the same blanks were prepared as above.

The rate of the hydrolysis of the substrates (amino acid 2-naphthylamides) used has been in most experiments expressed as extinction or as the change of molarity of 2-naphthylamine per min.

10. Thin layer chromatography. The thin layer chromatographic experiments were carried out with the Desaga devices (C. Desaga GmbH, 69 Heidelberg, Germany) on 0.35 mm thick Silica Gel G layers. The development was conducted with butanol-acetic acid-water mixture (3:1:1, by vol.), and the amino acids were detected with a modified ninhydrin reagent of Moffat and Lytle.[19] Otherwise the instructions given by Randerath [20] were followed. The chromatograms were scanned with the Chromoscan recording and intergrating densitometer equipped with a thin layer attachment (Joyce, Loebl & Co. Ltd., Gateshead, England).

11. Disc electrophoresis. The disc electrophoretic studies were carried out with the Canalco Model 12 apparatus (Canal Industrial Corp., Bethesda, Md., USA) following the suggestions and instructions of Ornstein and Davis for standard procedure. Consequently, the proteins have been resolved using the pH 9.5 gel (7 %) stained and fixed in 0.7 % Amidoschwarz 10 B in 7% acetic acid. The localization of enzyme activity in the gels was tested by incubating 0.1 ml of the protein solution extracted from 1 mm thick gel slabs in the usual reaction mixture presented earlier for iminopeptidase determination. The extraction was carried out from the 1 mm gel slabs for 3 h with 0.3 ml of 0.1 M tris-HCl buffer (pH 7.0) in an ice bath. Preliminary experiments had showed that the enzyme was somewhat inactivated if the whole gel column was incubated in the presence of the Fast Garnet GBC Salt, which contained stabilizers inhibiting many enzymes.

12. Determination of enzyme kinetic constants. The Michaelis constant, K_m, was determined by three different graphical methods: Lineweaver and Burk,[21] Eadie,[22] and Hanes.[23] In those cases where inhibition constants could be determined for the chemical compounds tested, this was done according to the method of Dixon.[24]

Table 2. Hydrolysis of L-prolyl-2-naphthylamide by various commercial enzyme preparations and by those obtained from the human oral cavity. Results are given as liberated μmoles of 2-naphthylamine per min and mg protein ($\times 10^6$). At pH 5.0 the experiments had been carried out in 0.025 M β,β-dimethylglutaric acid buffer, at pH 7.0 and 8.0 in 0.025 M phosphate buffer, and at pH 9.0 in 0.025 M boric acid-borax buffer. The zeros mean that no hydrolysis of the substrate involved was observed during the 60 min incubation time and under the conditions given.

Enzyme preparation	pH 5.0	pH 7.0	pH 8.0	pH 9.0
Leucine aminopeptidase	0	7.0	21.3	13.8
Trypsin	0	2.1	0	0
Chymotrypsin	0	0	0	0
Papain	0.3	0.9	0.9	0.8
Subtilisin	0	0.02	0.03	0
Supernatant fluid of whole saliva	2.0	4.0	4.0	3.1
Parotid saliva	2.0	3.0	3.0	2.0
Whole saliva sediment (Bacterial Plaque I)	14.0	22.0	23.2	22.0
Bacterial plaque extract (Bacterial Plaque II)	85.0	70.5	68.0	45.0
Aqueous extract of carious dentine	55.0	16.0	12.2	1.1
Aqueous extract of normal dentine	0	0.3	0.1	0

RESULTS

1. Hydrolysis of L-prolyl-2-naphthylamide by various enzyme preparations

Table 2 shows the results of studies on the ability of various enzyme preparations, derived from the human oral cavity, to hydrolyze L-prolyl-2-naphthylamide. Although even parotid glands seemed to liberate enzymes possessing proline iminopeptidase activity into the oral cavity, it was apparent that most of this activity, generally observed in the mouth, had originated in the micro-organisms of the bacterial plaque covering the teeth. The highest enzyme activity was almost constantly found in the aqueous extract of that bacterial plaque detached from the surface of the teeth by means of an excavator (Bacterial Plaque II). Table 2 also reveals the finding of considerable proline iminopeptidase activity in carious dentine, an indication that oral micro-organisms had penetrated deep into the dentinal tubules and had liberated enzymes therein, or perhaps that micro-organisms outside the carious lesion had liberated proline iminopeptidase which had then diffused into the dentine. Histochemical and other enzyme studies on carious dentine carried out in this laboratory support more strongly the first mentioned hypothesis.[25,26] Also normal dentine revealed some proline iminopeptidase activity. This may have represented enzyme activity normally found in dentinal fluid, but may also result from bacterial contamination. The latter possibility can partially

explain proline iminopeptidase activity of parotid saliva. With the method used here for collecting parotid saliva, it was almost impossible to avoid some bacterial contamination since the ducts of the glands constantly contain micro-organisms. For this reason, investigations of proline iminopeptidase activity in fluids from other salivary glands were not attempted.

When the enzyme activity of the above preparations was compared with that of the five commercial enzyme preparations mentioned in Table 2, it was seen that only the leucine aminopeptidase possessed significant proline iminopeptidase activity. This, and the lower enzyme activity of the other commercial preparations, may, of course, have been due to their insufficient purity. As illustration, the leucine aminopeptidase preparation was found, in certain experiments not described here, to catalyze the hydrolysis of most amino acid 2-naphthylamides commercially available, as well as the hydrolysis of various 2- and 1-naphthyl esters. Further, disc electrophoretic experiments, performed at pH 9.0 according to Ornstein and Davis, showed that the leucine aminopeptidase preparation being used was divided into at least fourteen bands, stainable with Amido Black. This further indicates that the enzyme preparation was not homogeneous (the other four enzyme preparations behaved practically homogeneously). However, the hydrolysis of the substrate by the leucine aminopeptidase preparation may indeed have been due to the action of the leucine aminopeptidase itself, since the highest rate of hydrolysis was always observed at alkaline pH values where this enzyme possesses its pH optimum for most substrates.[27]

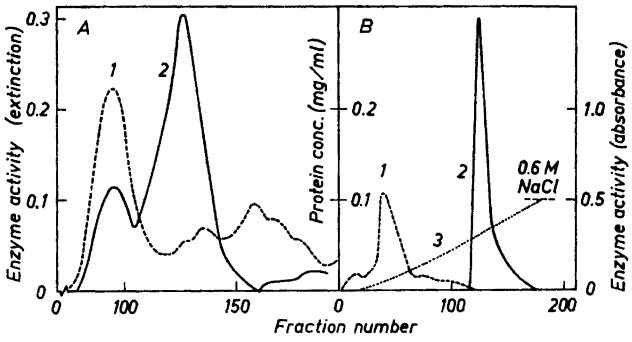

Fig. 1. Separation of proline iminopeptidases of human whole saliva supernatant fluid on a Sephadex G-200 column (A) and subsequent fractionation of the pooled and concentrated active fractions from this filtration on a DEAE-cellulose column (B). More detailed explanation of the preparation of the samples applied on the columns is given in that part of the text presenting a purification procedure for one proline iminopeptidase of human whole saliva. *Gel filtration:* Column: 4.5 × 110 cm; Elution buffer: 0.05 M tris-HCl buffer, pH 7.0; Flow rate: 0.4 ml/min; Hydrostatic pressure: 20 cm; fraction volume: 6 ml. Fractions 118–136 were pooled. *DEAE chromatography:* Column: 2.1 × 33.0 cm; Height of DEAE-cellulose filling approx. 31.5 cm at the start of the experiment; Elution buffer: 0.01 M tris-HCl buffer, pH 7.0, containing a sodium chloride gradient from 0 to 1.0 M; Mixing volume: 150 ml + 150 ml; Flow rate: approx. 0.1 ml/min; Hydrostatic pressure: 100 cm; Fraction volume: 1.5 ml. Fractions 121–130 were pooled. In both fractionations the protein concentration and the enzyme activity had been estimated from every fraction. 1: protein concentration; 2: enzyme activity; 3: NaCl concentration.

2. Fractionation of proline iminopeptidases

In order to determine the number of different proline iminopeptidases in the human oral cavity, the following chromatographic experiments were carried out.

a) Fractionation of whole saliva supernatant fluid through Sephadex G-200 columns. Two main enzyme peaks were obtained (Fig. 1A). When the fractions forming the second and more active enzyme peak were pooled and the resultant solution was concentrated and fractionated on DEAE-cellulose columns, only one proline iminopeptidase peak was obtained (Fig. 1B). (The method for the concentration of the protein solution is given later in the section presenting a purification procedure for one proline iminopeptidase). This single enzyme peak was later shown to correspond to the third proline iminopeptidase peak obtained when fractionating the supernatant fluid of whole saliva directly on a DEAE-cellulose column, without a preceding gel filtration. It also corresponded in all enzyme properties studied to an enzyme obtained by fractionating a protein solution, obtained in turn from gel filtration experiments on Sephadex G-100, on the same DEAE-cellulose column (Fig. 2). The first enzyme peak from Sephadex G-200 filtrations was shown to correspond to the second proline iminopeptidase peak obtained later in all the DEAE-cellulose fractionations in this study. In repeated experiments, the relative ratio of the three proline iminopeptidase-like enzymes found in fractionation experiments varied, a phenomenon evidently dependent on conditions in the oral cavity of the person who had supplied the test material (see Fig. 3, as an illustration of this variation).

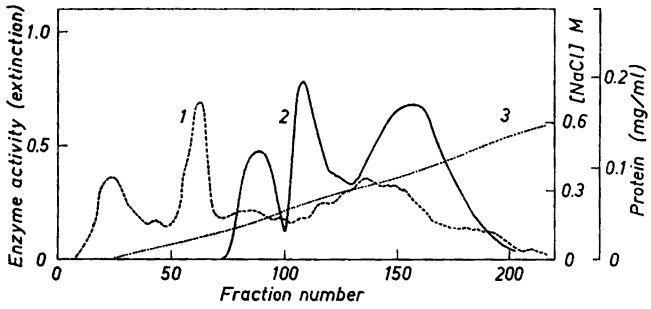

Fig. 2. DEAE-cellulose fractionation of proline iminopeptidases from an enzyme preparation obtained by first fractionating whole saliva supernatant fluid on a Sephadex G-100 column. The enzymatically active fractions from this filtration had been then pooled and concentrated as presented in the Methods section. Column: DEAE-cellulose, 1.5 × 20.0 cm; Height of DEAE-cellulose filling approx. 18 cm at the start of the experiment. Sample: 15 ml of a protein solution which had been obtained by passing 7 ml of an enzyme preparation (obtained by molecular exclusion chromatography on Sephadex G-100) through a Sephadex G-25 column (2.0 × 25.0 cm) with the aid of 0.01 M tris-HCl buffer, pH 7.2. 0.3 ml of 0.2 % Blue Dextran solution was added to the solution applied on the G-25 column and 15 ml was collected from the column; Elution buffer: 0.01 M tris-HCl, pH 7.2, containing a NaCl gradient from 0 M to 1.0 M; Flow rate: appr. 0.1 ml per min; Mixing volume: 150 ml + 150 ml; Hydrostatic pressure: 100 cm; Temperature: 1°C. 1: protein concentration; 2: enzyme activity; 3: NaCl concentration.

b) Fractionation of whole saliva supernatant fluid through Sephadex G-100 columns. Only one main enzyme peak was observed. When this was further fractionated on DEAE-cellulose column, three separate proline iminopeptidase peaks were obtained (Fig. 2). The proline iminopeptidase fractionated last from the column corresponded to the only enzyme peak in Fig. 1B.

c) Fractionation of the supernatant fluid of whole saliva and its sediment, treated with ultrasonic disintegrator, on DEAE-cellulose columns. Three separate enzyme peaks were constantly observed. These were shown to correspond to the three enzymes obtained earlier in DEAE-cellulose chromatography (with a preceding filtration through Sephadex G-100), shown in Fig. 2.

The chromatographic experiments had revealed three separate enzymes possessing proline iminopeptidase activity, regardless of the origin of the test material: whole saliva supernatant fluid, or the sediment obtained after the centrifugation, the sediment having been treated with ultrasonic sound. These results provided the strong possibility that all of the three enzymes possessing proline iminopeptidase activity had been formed in the micro-organisms of the bacterial plaque, from which they had been liberated into the saliva, either as the result of an active secretion or of cell death.

It was stated earlier that the mutual ratio of the activity of the three proline iminopeptidase preparations varied in repeated fractionation experiments. This is illustrated also in Fig. 3 which gives the limits of the activity of the enzymes in four separate fractionations. The material (whole saliva supernatant fluid) had come from different collections.

Fig. 4 shows the behaviour of two proline iminopeptidase preparations (those fractionated later from the DEAE-cellulose columns) on disc electrophoresis gels. These enzyme preparations were obtained from the supernatant fluid of whole saliva sediment (treated with ultrasonic disintegrator). Only one enzymatically active area was observed in the gels, whereas several protein bands were seen, an indication of considerable crudeness. The same kind of fractionation patterns were obtained with enzyme preparations from the supernatant fluid of whole saliva when the location of the proline iminopeptidases in the gels was determined (the proteins, of course, displayed a different pattern).

3. Purification of one proline iminopeptidase from whole saliva supernatant fluid

A more detailed study of the most active of the three proline iminopeptidases in the human oral cavity, or that occurring in highest amount in the material studied, (the same whose fractionation on DEAE-cellulose is given in Fig. 1B) was made in order to develop a simple procedure for its purification. The following procedure was developed.

1) All steps in the purification procedure were carried out at 0—4°C unless otherwise stated, and all centrifugations were conducted at 19 500 rpm (45 900 *g*) for 10 min.

2) Whole saliva from one person (male) with open caries lesions was collected by stimulating the secretion with paraffin. Portions of 50 ml were collected daily. The material was allowed to run into a 50 ml plastic bottle placed in an

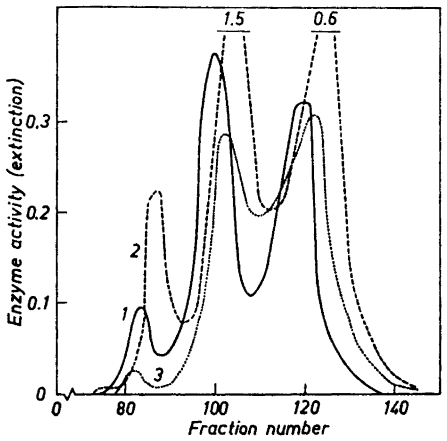

Fig. 3. Separation of proline iminopeptidases of the supernatant fluid of human whole saliva on DEAE-cellulose columns. The figure presents three separate fractionations for which the sample applied had been obtained from different collectings of saliva. The aim of the figures is to illustrate the variation of the height of the three proline iminopeptidase peaks in different experiments. Details for the fractionations are the same as for Fig. 2. 1, 2, and 3 refer to the different chromatograms, determined with L-prolyl-2-naphthylamide.

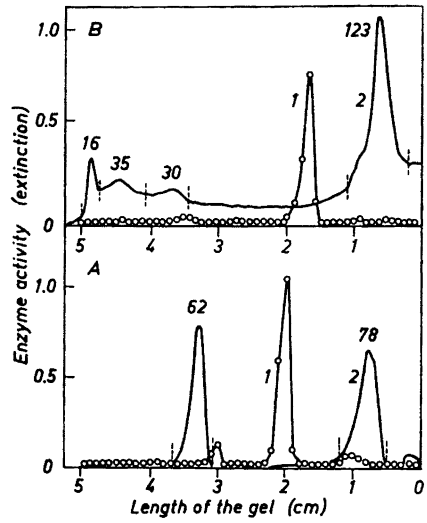

Fig. 4. Separation of two proline iminopeptidases (those fractionated later from the DEAE-cellulose columns) of human whole saliva sediment, (Bacterial Plaque I, treated with ultrasonic disintegrator as presented in the Methods section [15]) on polyacrylamide columns at 22°C (current: 1.5 mA per gel). 1: enzyme activity; 2: densitometric trace of the sample. Position, abscissa; Optical density, ordinate, 2 OD full-scale (the whole height of the figures A and B correspond to 2 OD). A: proline iminopeptidase II; B: proline iminopeptidase III. The figures above the peaks, marked by vertical lines, illustrate contaminating protein bands and describe their relative intensity, determined with an integrating densitometer, employing a special Chromoscan filter for Amido Schwarz. The direction of the current is from the right to the left.

ice bath, centrifuged (Supernatant Fluid I was obtained) and frozen immediately afterwards and stored at about −20°C until used (about ten days).

3) After thawing, the 50 ml samples of saliva were pooled (500 ml) and centrifuged (Supernatant Fluid II was obtained). The sediment was discarded. The volume of the solution (495 ml) was decreased by bringing it up to 80 % saturation with ammonium sulphate (Special Enzyme Grade, Mann Research Laboratories) by dissolving the dry salt in the protein solution. The mixture was allowed to stand for 1 h and centrifuged. The supernatant fluid was discarded.

4) The precipitate was suspended in 45 ml of cold water and the undissolved portion was spun down (Supernatant Fluid III was obtained).

5) The resultant solution of 41 ml was allowed to stand for 48 h and the formed turbidity was removed by centrifugation.

6) The resultant, nearly 41 ml protein solution was passed through a Sephadex G-200 column and the fractions forming the enzyme peak hydrolyzing L-prolyl-2-naphthylamide were pooled to form a crude proline iminopeptidase solution of 110 ml.

7) The volume of this solution was reduced by bringing it up to 80 % saturation with ammonium sulphate as above (Supernatant Fluid IV was obtained). (The earlier mentioned concentrating of samples to be applied on DEAE-cellulose columns was performed in a similar way as described in steps 6 and 7.)

8) The resultant aqueous proline iminopeptidase solution of 6.2 ml was fractionated through a DEAE-cellulose column (for detail see Fig. 1B) and the most active fractions were pooled to form a 15 ml solution of the enzyme. This enzyme preparation was stored during the experiments (eight weeks) at 4°C without any apparent changes in its activity.

For DEAE fractionation, no preceding transfer of the crude enzyme preparation to the ionic strength of the initial buffer was carried out, since it was found that the enzyme was very strongly attached to DEAE-cellulose. The enzyme required almost 0.5—0.6 M NaCl concentration in the column to be completely detached from the cellulose. The details of the fractionations with Sephadex G-200 and DEAE-cellulose were the same as given in Fig. 1, when smaller amounts of saliva were used as starting material in preliminary experiments. In spite of different amounts of material, the preparative purification procedure gave the same elution patterns in column chromatography as in the preliminary experiments. The purification procedure is summarized in Table 3. The enzyme preparation was homogeneous in rechromatography on Sephadex G-200 and DEAE-cellulose. On the other hand, disc electrophoretic studies with 20 μl amounts of the final enzyme preparation showed that it was divided into several faint protein fractions, although only one enzymically active region was observed (Fig. 4). The results in disc electrophoresis were very

Table 3. Purification of a proline iminopeptidase from human whole saliva. The enzyme activity is expressed as the amount of μmoles of 2-naphthylamine liberated in the reaction mixture per min and mg protein (in 0.025 M phosphate buffer, pH 7.0, at 37°C). Details in performing the individual purification steps are presented in the text.

Step	Activity ($\times 10^3$)
Supernatant fluid I	0.11
Supernatant fluid II	0.21
Supernatant fluid III	2.10
After G-200 filtration	3.70
Supernatant fluid IV	5.60
After DEAE cellulose chromatography	67.00

similar to those obtained with the third proline iminopeptidase preparation, presented in the same figure.

The purity of the enzyme preparation was considered satisfactory for most enzyme studies. It was found, however, in testing the reproducibility of the presented method that the procedure was markedly improved by more effective utilization of the strong attaching qualities of the enzyme to DEAE-cellulose, as well as by carrying out ammonium sulphate fractionation instead of simple precipitation.

4. Substrate specificity

Although the proline iminopeptidase preparations obtained by single DEAE-cellulose chromatography, and even the more thoroughly purified enzyme, still contained other proteins, it was found necessary to study the

Table 4. Hydrolysis of 2-naphthylamides (2-NA) of various amino acids by the three enzyme preparations obtained by DEAE-cellulose chromatography on an enzyme preparation made from whole salive sediment (Bacterial Plaque I) with ultrasonic disintegrator as described in the Methods section, and by the more thoroughly purified enzyme (IV). The experiments had been carried out for 60 min in 0.025 M phosphate buffer, pH 7.0, with 1.66×10^{-4} M substrate concentration. The rates of the hydrolyses are given in liberated μmoles of 2-naphthylamine per min and mg protein ($\times 10^3$). The zeros mean that no hydrolysis in the cases given was observed.

Substrate	I	II	III	IV
L-Alanyl-2-NA	2.18	1.80	0.82	0
L-Arginyl-2-NA	0.81	1.90	0.69	0
L-Asparagyl-2-NA	0.08	0.22	0.16	0
L-α-Aspartyl-2-NA	0.09	0.72	0.31	0
L-β-Aspartyl-2-NA	0.03	0.16	0	0
L-Cystine di-2-NA	0.02	0.16	0	0
L-γ-Glutamyl-2-NA	0.06	0.33	0.12	0
L-β-Glutamyl-2-NA	0.12	0.72	0.29	0
Glycyl-2-NA	0.37	0.57	0.12	0
L-Histidyl-2-NA	0.06	0.22	0	0
L-Hydroxyprolyl-2-NA	0.09	3.24	2.77	8.5
L-Isoleucyl-2-NA	0.12	0.16	0	0
L-Leucyl-2-NA	1.10	0.19	1.54	0
L-Leucyl-4-methoxy-2-NA	0.80	0.92	0.20	0
L-Lysyl-2-NA	0.60	1.30	0.29	0
L-Methionyl-2-NA	1.24	1.00	0.55	0
N-α-Benzoyl-DL-arginyl-2-NA	0.05	0.06	0	0
L-Ornityl-2-NA	0.16	0.50	0	0
L-Phenylalanyl-2-NA	0.65	1.90	4.20	0
L-Prolyl-2-NA	0.28	19.02	7.00	67.3
L-Seryl-2-NA	0.15	0.37	0	0
L-Threonyl-2-NA	0.14	0.14	0	0
N-α-Tosyl-L-arginyl-2-NA	0.02	0.10	0	0
L-Tryptophyl-2-NA	0.08	0.37	0.22	0
L-Tyrosyl-2-NA	0.11	0.26	0.22	0
L-Valyl-2-NA	0.11	0.20	0	0

relative ability of the enzyme preparations to hydrolyze 2-naphthylamides of various amino acids, as well as some peptides, in particular those containing *N*-terminal proline. The results of all the substrate specificity studies are given in Table 4 and in Fig. 5. In Table 4, the ability of various enzyme prepara-

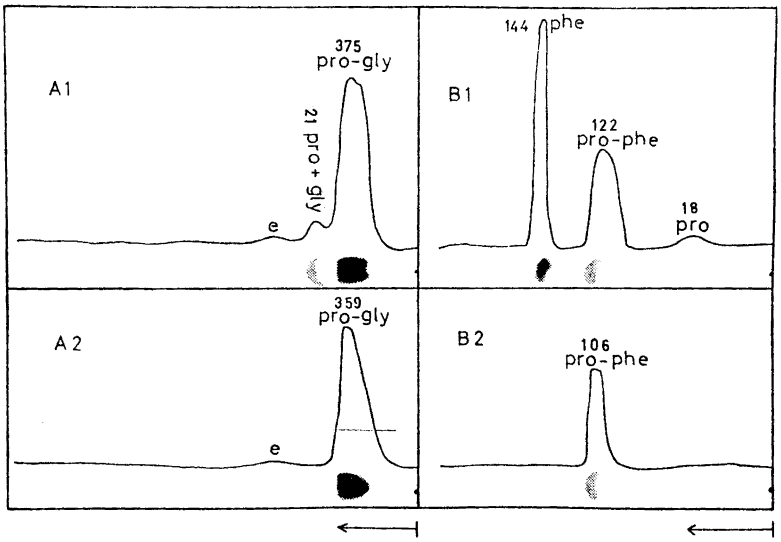

Fig. 5. Demonstration of the hydrolysis of L-prolylglycine (A) and L-prolyl-L-phenylalanine (B) by the purified proline iminopeptidase preparation. The spots represent the substrates and the products of their hydrolysis, separated by thin layer chromatography, and stained with ninhydrin, as explained in the Methods section. Solid line: densitometric trace of the chromatogram. Position, abscissa; Optical density, ordinate, 2 OD full scale (the height of the figures correspond to 1 OD). A1 and B1: reaction in the presence of enzyme; A2 and B2: substrate blank (omitting the enzyme preparation). The enzyme preparation used gave no visible or only faint reaction with ninhydrin. Pro-gly=L-prolylglycine; pro-phe=L-prolyl-L-phenylalanine; e=enzyme. The figures above the peaks illustrate their relative intensity, determined with an integrating densitometer, employing blue green filter (Ilford 601, peak transmission 5220 Å). The low figures in the substrate blank experiments are due to the weakening of the intensity of the spots (in spite of using a modified ninhydrin reagent) while scanning a larger number of plates of other reaction mixtures.

tions to hydrolyze various amino acid 2-naphthylamides is presented. The enzyme preparation fractionated first from the DEAE-cellulose column seemed to contain an enzyme with lower substrate specificity, or else it contained enzymes denoted more correctly as amino-, and not iminopeptidases.

The two other enzyme preparations fractionated last from the DEAE-cellulose column seemed to be more "iminopeptidase-like" by their substrate specificity. It is to be noted that each preparation was also able to hydrolyze L-hydroxyprolyl-2-naphthylamide at a considerable rate and that the third preparation cleaved L-phehylalanyl-2-naphthylamide at a significant rate as well. This latter hydrolysis may have been caused also by contaminating

enzymes. When this proline iminopeptidase was purified more thoroughly, only the 2-naphthylamides of L-proline and L-hydroxyproline were hydrolyzed. The purified proline iminopeptidase preparation from whole saliva was able to hydrolyze also some peptides (Fig. 5). The substrates selected to represent various types of compounds which were thought to be susceptible to hydrolysis by the purified enzyme were: glycyl-L-proline, L-prolylglycine, L-hydroxyprolylglycine, glycyl-L-prolylglycylglycine, glycylglycyl-L-hydroxyproline and L-prolyl-L-phenylalanine. As shown in Fig. 5, only L-prolyl-L-phenylalanine and L-prolylglycine were hydrolyzed at a significant rate. It is evident that the aromatic nucleus of phenylalanine, close to the peptide bond, causes an increase in the rate of the enzymic hydrolysis. Hence it is possible that the earlier observed cleavage of L-phenylalanyl-2-naphthylamide by the third enzyme peak was indeed due to the action of an iminopeptidase and not to the action of impurities (Table 4). Because the purified enzyme was not found to hydrolyze this substrate, the observed reaction in Table 4 may have been due to some contaminating enzyme in the crude enzyme preparation. However, the observed cleavage of L-prolyl-L-phenylalanine (Fig. 5) suggests that the purified proline iminopeptidase in question did not hydrolyze only imino-acid-2-naphthylamides, but *N*-terminal proline peptides as well. The free imino group seems to be necessary for the reaction. When poly-L-hydroxyproline, poly-L-proline, and poly-*O*-acetyl-L-hydroxyproline were used as substrate, no liberation of free proline, hydroxyproline or shorter peptides was observed by the methods used (thin layer chromatography).

5. Kinetics of the enzyme reactions

Hydrolysis of L-prolyl-2-naphthylamide by the purified enzyme as well as by all of the three enzyme preparations obtained by mere DEAE-cellul se fractionations, was found to be linear with time. This is shown with the first mentioned enzyme preparation in Fig. 6. When the two substrates, L-prolyl-2-naphthylamide and L-hydroxyprolyl-2-naphthylamide, were added together at the same concentration, the total rate of the reaction was always less than the sum of the rates of the reactions measured separately (it was ascertained that the substrate concentrations used were sufficient to saturate or nearly saturate the enzymes when added separately). The hydrolysis of L-prolyl-2-naphthylamide was inhibited by the corresponding amide of L-hydroxyproline. This result also indicated that both of the substrates had indeed been hydrolyzed by one and the same enzyme. Quite similar results were obtained with all of the three enzyme preparations from DEAE-cellulose chromatography, regardless of their origin (Bacterial Plaque I and whole saliva).

The rate of the hydrolysis of L-prolyl-2-naphthylamide was in each case found to be directly proportional to the amount of enzyme present in the reaction mixture, as shown in Fig. 7. The figure shows results with enzyme preparations obtained from whole saliva supernatant fluid. Similar results were again obtained with enzymes of whole saliva sediment (Bacterial Plaque I), treated with ultrasonic disintegrator, and with the purified enzyme as well. Dependence of the rate of the hydrolysis on the substrate concentration at pH

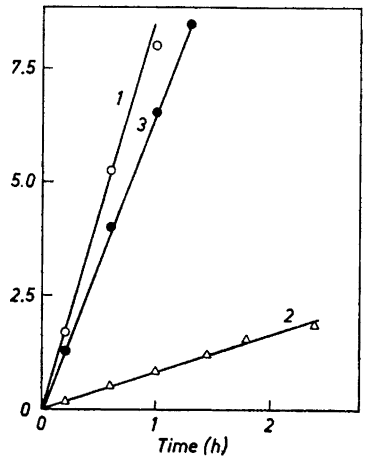

Fig. 6. Rate ($\times 10^7$ M min^{-1}) of hydrolysis by the purified proline iminopeptidase of L-prolyl-2-naphthylamide (1) and L-hydroxyprolyl-2-naphthylamide (2) alone and combined (3). Concentration of each substrate: 0.166×10^{-3} M. Tested in 0.025 M phosphate buffer, pH 7.0.

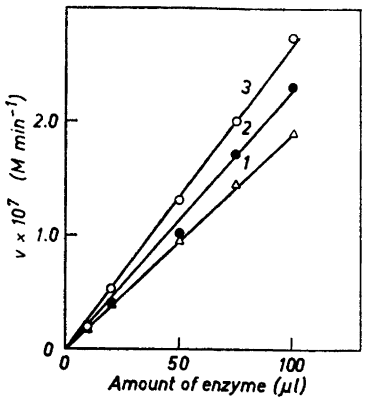

Fig. 7. Dependence of the rate of the hydrolysis of L-prolyl-2-naphthylamide on the quantity of the enzyme in 0.025 M phosphate buffer, pH 7.0. Substrate concentration: 0.166×10^{-3} M. The three enzyme preparations used had been obtained by fractionating the supernatant fluid of human whole saliva on a DEAE-cellulose column. 1: proline iminopeptidase I; 2: proline iminopeptidase II; 3: proline iminopeptidase III.

7.0, in 0.025 M phosphate buffer, is presented in Fig. 8 with the three enzyme preparations obtained from whole saliva supernatant fluid. Under the conditions given a saturation of the enzymes was obtained at a concentration of about 0.04—0.08 mM. At higher substrate concentrations substrate inhibition began to occur. These results were obtained by plotting the experimental data according to Lineweaver and Burk. Essentially similar results were obtained when the method of Eadie or that of Hanes was employed.

The distribution of the experimental points in Fig. 8 is such that a certain error may have occurred in calculating the Michaelis constant for the three cases. This error is due to substrate inhibition. The constants determined may include another constant, describing the inhibition involved. However, even the results of Fig. 8 revealed that the enzyme preparations numbered II and III resembled each others in the K_m values calculated for the hydrolysis of L-prolyl-2-naphthylamide. The enzyme preparation called I differed clearly from the former in the magnitude of the K_m value.

Also the purified proline iminopeptidase was studied in the presence of different concentrations of L-prolyl-2-naphthylamide, but in this case at several pH values. Results are given in Fig. 9. The kinetics of the hydrolysis was found to be fairly "normal" in the sense that the substrate-velocity curves

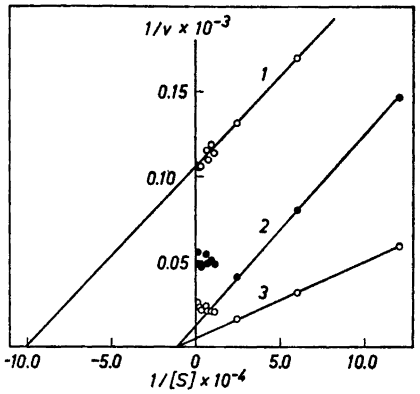

Fig. 8. Effect of high substrate (L-prolyl-2-naphthylamide) concentration on the action of the three proline iminopeptidase preparations, obtained by fractionating supernatant fluid of human whole saliva on a DEAE-cellulose column. The values of K_m, determined graphically according to the method of Lineweaver and Burk, were approximately 1.0×10^{-3} M, 1.0×10^{-4} M, and 1.0×10^{-4} M, respectively, for the three enzyme preparations. As to the effect of high substrate concentrations on these graphically determined constants, see text. 1: proline iminopeptidase I; 2: proline iminopeptidase II; 3: proline iminopeptidase III. Tested in 0.025 M phosphate buffer, pH 7.0. In this case the rate of the hydrolysis was measured as liberated μmoles of 2-naphthylamine per min and mg protein.

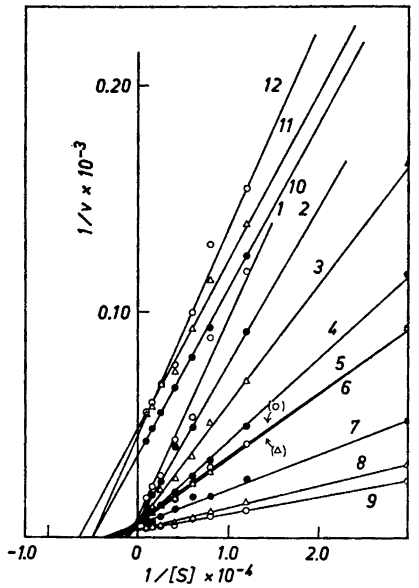

Fig. 9. Effect of pH and substrate (L-prolyl-2-naphthylamide) concentration on the action of the more thoroughly purified proline iminopeptidase (III) of human whole saliva supernatant fluid. The experiments had been performed in 0.025 M phosphate buffer. 1=pH 5.8; 2=pH 6.0; 3=pH 6.2; 4=pH 6.4; 5=pH 6.6; 6=pH 6.8; 7=pH 7.0; 8=pH 7.2; 9=pH 7.4; 10=pH 7.6; 11=pH 7.8; 12=pH 8.0.

yielded the expected hyperbola and the double-reciprocal plots gave straight lines. The values for the apparent Michaelis constant with the purified proline iminopeptidase were not very low, an indication to a fairly weak binding between the substrate and enzyme.

The kinetic experiments already described had been carried out in 0.025 M phosphate buffer. The effect of the substrate concentration on the hydrolysis of L-prolyl-2-naphthylamide by the purified enzyme was studied also in 0.025

M boric acid-borax buffer. In this buffer the values of the apparent Michaelis constant were very nearly the same as those obtained in phosphate buffer. Boric acid-borax buffer, however, inhibited the hydrolysis of the substrate to a certain extent, the rate of the hydrolyses being approximately 75 % of that observed with phosphate buffer.

Because it was found that the substrate concentration of 1.66×10^{-4} M was high enough to saturate or at least nearly saturate the enzymes involved at pH 7.0, in various buffers, this substrate concentration was used when studying the effect of hydrogen ion concentration on the reactions in more detail. In this connection it was realized, however, that the substrate concentration used may not have been fully saturating the enzymes at all pH values studied. The results are shown in Fig. 10. The experiments were carried out in two different buffers and with all of the three enzyme preparations obtained by DEAE-cellulose chromatography from the supernatant fluid of

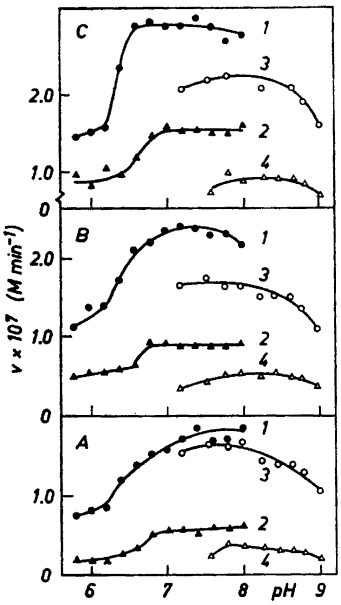

Fig. 10. Effect of pH on the hydrolysis of L-prolyl-2-naphthylamide by the three enzyme preparations obtained from the supernatant fluid of human whole saliva by DEAE-cellulose chromatography. 1 and 2: L-prolyl- and L-hydroxyprolyl-2-naphthylamide, respectively, tested in 0.025 M phosphate buffer; 3 and 4: the same substrates tested in 0.025 M boric acid-borax buffer. A: proline iminopeptidase I; B: proline iminopeptidase II; C: proline iminopeptidase III.

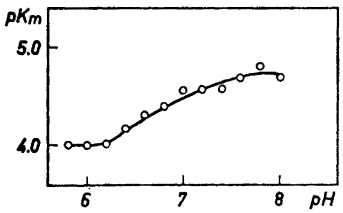

Fig. 11. Effect of pH on K_m in the hydrolysis of L-prolyl-2-naphthylamide by the more thoroughly purified proline iminopeptidase (III) in 0.025 M phosphate buffer. The values of K_m had been determined graphically by the method of Lineweaver and Burk.[21]

whole saliva, or from its sediment (results with the former starting material are shown). With the purified enzyme preparation the results were quite similar to those obtained with the proline iminopeptidases called II and III, and are therefore not shown. The effect of pH on the hydrolyses was almost the same, regardless of the origin of the enzymes (whether whole saliva supernatant fluid or Bacterial Plaque I). Also these results showed the inhibitory effect of boric acid-borax buffer. The use of boric acid-borax buffer was also found to shift the pK_m *versus* pH curves to the right. The rate of the hydrolyses of L-hydroxyprolyl-2-naphthylamide was fairly low and even this property of the various enzymes was the same regardless of the origin of the enzymes.

In Fig. 11 the effect of hydrogen ion concentration on the Michaelis constant is given for the hydrolysis of L-prolyl-2-naphthylamide by the purified enzyme. The values for K_m were obtained from the plots of Lineweaver and Burk. When examining the curve in Fig. 11, one could assume that the upward bend at pH 6.2 corresponds to the pK of a group of the enzyme-substrate complex. The bend at about pH 7.0 could in turn represent the pK of ionizing groups in one of the components of the reacting system.

6. Effect of chemical compounds

The effect of several chemical compounds on the action of the purified proline iminopeptidase from human whole saliva was studied. In addition, enzyme preparations from mere DEAE-cellulose fractionations were also studied, although it was realized that higher amounts of contaminating proteins in the reaction mixtures may interfere with kinetic inhibition studies. Many of the chemical compounds tested had only little effect on the rate of the hydrolysis of L-prolyl-2-naphthylamide. Hence no K_i values could reasonably be calculated for most of the compounds tested, but only percentual inhibition. The most interesting results were: *N*-ethylmaleimide and sodium *p*-chloromercuribenzoate, usually inhibiting the action of so-called SH-enzymes, and L-cysteine and dithiothreitol, usually activating such enzymes, were without effect, when the proline iminopeptidases II and III were studied. The enzyme preparation called I differed clearly from the two other preparations: it was inhibited by *N*-ethylmaleimide and *p*-chloromercuribenzoate. The only inhibiting compound tested with II and III was diphenylcarbamyl chloride, indicating that these two iminopeptidases may possess some similarity in their action to chymotrypsin or trypsin which are specifically inhibited by this reagent.[28,29,33] An important result was also the effect of EDTA: it did not not cause any activation or inhibition.

The graphical determination of K_i values for diphenylcarbamyl chloride, sodium *p*-chloromercuribenzoate and *N*-ethylmaleimide (in those cases where they had caused inhibition) was performed according to Dixon.[24] The K_i values obtained for these compounds were: diphenylcarbamyl chloride, 2.8×10^{-4} M, 3.2×10^{-4} M, and 1.5×10^{-4} M, for the enzyme preparations I, II, and III, respectively. These values represent experiments with the DEAE-

Table 5. Effect of various chemical compounds on the hydrolysis of L-prolyl-2-naphthylamide by the three enzyme preparations obtained by DEAE-cellulose chromatography on the supernatant fluid of human whole saliva, and its sediment (Bacterial Plaque I), the latter treated with ultrasonic disintegrator. The experiments had been carried out for 3 h in 0.025 M phosphate buffer, pH 7.0, without any preincubation. Figures represent percentage inhibition, 0 being assigned to conditions without no marked effect on the rate of the hydrolysis. The experiments had been carried out as three or four separate sets and the figures are given as mean values from these experiments at 5 % accuracy. I, II, and III refer to the three proline iminopeptidases + mean activation.

Compound tested	Enzyme preparation	Concentration of the chemical compound (M) × 10^4											
		16.6	8.30	1.66	0.83	0.166	0.083	16.6	8.30	1.66	0.83	0.166	0.083
		DEAE peaks from whole saliva supernatant fluid						DEAE peaks from whole saliva sediment					
p-Toluenesulphonyl-L-phenylalanylchloromethyl ketone	I	15	15	0	0	0	0	20	15	10	0	0	0
	II	15	15	0	0	0	0	25	20	10	0	0	0
	III	0	0	0	0	0	0	15	15	10	0	0	0
Nα-Tosyl-L-lysyl chloromethane	I	15	10	0	0	0	0	20	15	10	0	0	0
	II	15	10	0	0	0	0	15	15	10	0	0	0
	III	10	10	0	0	0	0	20	15	10	0	0	0
N-Ethylmaleimide	I	75	60	40	30	30	30	85	70	50	35	25	15
	II	25	20	10	0	0	0	10	5	0	0	0	0
	III	20	10	10	5	0	0	10	5	5	0	0	0
p-Chloromercuribenzoate	I	100	85	85	75	75	75	100	100	85	85	75	75
	II	15	10	10	10	10	10	5	0	0	0	0	0
	III	10	10	10	5	5	5	10	5	0	0	0	0
EDTA	I	0	0	0	0	0	0	10	5	5	0	0	0
	II	0	0	0	0	0	0	0	0	0	0	0	0
	III	0	0	0	0	0	0	0	0	0	0	0	0
Phenylmethanesulphonyl fluoride	I	35	35	10	10	5	0	30	30	10	0	0	0
	II	40	35	10	0	0	0	30	30	15	0	0	0
	III	30	25	0	0	0	0	35	30	10	0	0	0
Diphenylcarbamyl chloride	I	100	85	65	40	20	15	100	80	50	35	20	10
	II	100	80	60	45	25	15	95	75	50	35	20	15
	III	100	90	60	40	20	20	100	80	50	40	15	10
L-Cysteine	I	+15	+10	0	0	0	0	+20	+15	+10	+10	+10	+10
	II	10	10	0	0	0	0	10	0	0	0	0	0
	III	15	10	0	0	0	0	10	0	0	0	0	0
Dithiothreitol	I	+15	+15	+10	0	0	0	+25	+20	+20	+15	+10	+10
	II	0	0	0	0	0	0	0	0	0	0	0	0
	III	0	0	0	0	0	0	0	0	0	0	0	0

cellulose pools obtained by using the supernatant fluid of whole saliva as starting material. The corresponding values of K_i for enzymes obtained from the whole saliva sediment (Bacterial Plaque I) were very nearly the same as above. In addition, experiments with the purified enzyme yielded essentially similar K_i values for diphenylcarbamyl chloride as above: constantly close to 2.0×10^{-4} M. The K_i values for *N*-ethylmaleimide and sodium *p*-chloromercuribenzoate tested with the enzyme preparation called I, were 2.0×10^{-4} M, and 3.0×10^{-5} M, respectively. With the other chemical compounds this method of plotting [24] yielded curves parallel or nearly parallel with the *x*-axis, indicating that they had no effect on the enzymatic reactions. This result is also shown in Table 5.

The hydrolysis of L-prolyl-2-naphthylamide by the purified enzyme, as well as by the six other enzyme preparations (preparations I, II, and III, from whole saliva supernatant fluid, or from its sediment, treated with ultrasonic disintegrator) was inhibited by L-hydroxyprolyl-2-naphthylamide, as already mentioned (Fig. 6). It was further found that free L-proline and L-hydroxyproline at concentrations ranging from 0.83×10^{-5} M to 0.166×10^{-2} M did not inhibit the hydrolysis of the corresponding 2-naphthylamides (when the amide concentration had been 0.166×10^{-3} M). Hence it appeared that of the end products of the hydrolysis the free amino acids were not inhibitory. However, when intact molecules of L-hydroxyproline-2-naphthylamide were present in the reaction mixture, the hydrolysis of the L-proline derivative was retarded. This phenomenon can be described as a competitive inhibition between the two amides.

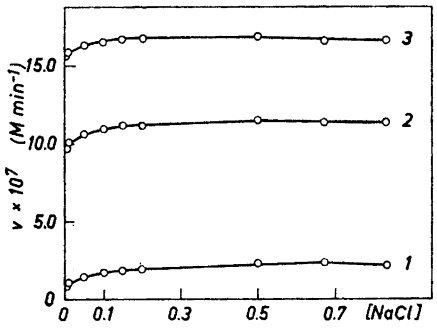

Fig. 12. Effect of NaCl (M) on the hydrolysis of L-prolyl-2-naphthylamide by the three proline iminopeptidase preparations obtained from human whole saliva supernatant fluid using DEAE-cellulose chromatography. 1, 2, and 3 refer to the three enzyme preparations (proline iminopeptidase I, II, and III, respectively). Tested in 0.025 M phosphate buffer, pH 7.0. Substrate concentration: 1.66×10^{-4} M.

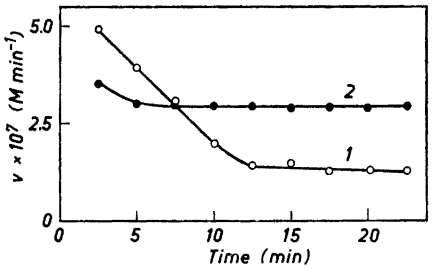

Fig. 13. Effect of collecting time on the proline iminopeptidase activity of samples (2.0 ml) of human whole saliva. The exact method of collecting is given in text. 1: enzyme activity; 2: protein concentration. Substrate: 1.66×10^{-4} M L-prolyl-2-naphthylamide.

Finally, it was tested if NaCl could increase the rate of the hydrolysis of L-prolyl-2-naphthylamide, using the purified enzyme and the three enzyme preparations obtained from DEAE-cellulose fractionations of whole saliva supernatant fluid. Results are given in Fig. 12 for the enzyme preparations designated I, II, and III. It was found that NaCl at the concentrations tested produced only a slight increase in the rate of the hydrolyses. The experiments with the purified proline iminopeptidase yielded results similar to those obtained with the proline iminopeptidase III. The form of the curves obtained was constantly different from that usually displayed, for example, by aminopeptidase B of rat liver. The latter enzyme is strongly activated in the presence of certain monovalent anions, in particular, chloride ions.[10,12,13]

7. Formation of proline iminopeptidases by bacteria

Because it was found that absolutely the most of all iminopeptidase activity in the human oral cavity originated in the micro-organisms of the dental plaque, or in those on oral epithelium, the ability of some micro-organisms to form enzymes capable of hydrolyzing L-prolyl-2-naphthylamide was studied. A proof on the occurrence of this enzyme activity on oral surfaces is yet given in Fig. 13. The enzyme preparations in this experiment were supernatant fluids of whole saliva samples, collected at proper time intervals. These preparations contained enzymes liberated most likely from oral micro-organisms. Whole saliva collected during the first few minutes by paraffin stimulation displayed higher proline iminopeptidase activity than the saliva collected, for example, after the first 10 min. Because the protein concentration of the samples decreased more slowly than the enzyme activity, this could indicate that the chewing of paraffin had rapidly removed most of the soft plaque cover on the teeth and microorganisms from other oral surfaces already during the first minutes of the experiment. Consequently, the most active proline iminopeptidase preparations could be obtained from the most easily removable plaque.

When the proline iminopeptidase production of the test organisms was studied, the enzyme activity was first calculated per ml of growth medium. Results are given in Table 6, where also the hydrolysis of 2-naphthylamides of some other amino acids is shown for the sake of comparison. In the same table the relative proline iminopeptidase content of the cells of the same test organisms in also presented. The most striking result was that the lactobacilli were able to form and liberate into their growth medium considerable amounts of enzymes possessing proline iminopeptidase activity. The *Corynebacterium bovis* which also occurs in the human mouth, possessed fairly high proline iminopeptidase activity, as well as the two strains of *E. coli*. The examples shown in Table 6 may provide sufficient proof for the assumption that the oral lactobacilli may be responsible for most of that proline iminopeptidase activity found in the human oral cavity. Then it must be realized that the conditions used in cultivating the test organisms in this study have by no means been comparable with those existing in the oral cavity. This means that

Table 6. Ability of various micro-organisms to liberate in their growth medium enzymes capable of hydrolyzing 2-naphthylamides of various L-amino acids (A) and ability of enzyme preparations made from the cells of various micro-organisms to hydrolyze the same substrates (B), in 0.025 M phosphate buffer, pH 7.0. The enzyme preparations for A were made by diluting the clear growth mediums ten-fold with cold (4°C) water just before use. Details from cultivations of the test organisms are given in Table 1 and elsewhere.[15] The enzyme preparations for B had been made from harvested cells as presented elsewhere.[15] The enzyme activity in A is expressed as liberated μmoles of 2-naphthylamine per min and ml ($\times 10^4$) clear and centrifuged growth medium devoid of bacterial cells. The enzyme activity in B is expressed as liberated μmoles of 2-naphthylamine per min and ml turbid growth medium ($\times 10^4$): *i.e.*, the formation of 2-naphthylamine in cells present in 1 ml growth medium is given. The abbreviations pro, ala, arg, leu, lys, and met refer to the corresponding 2-naphthylamides of L-proline, L-alanine, L-arginine, L-leucine, L-lysine, and L-methionine, respectively. The zeros in the table mean that no hydrolysis of the substrates involved was detected during the 24 h (A) or 5 h (B) incubation time used.

Organism	A						B					
	Pro	Ala	Arg	Leu	Lys	Met	Pro	Ala	Arg	Leu	Lys	Met
L. casei NCTC 10302	1.3	11.2	2.9	9.1	5.4	3.5	8.5	9.5	17.5	22.1	15.4	9.0
L. casei ssp. *rhamnosus* ATCC 7469	1.1	9.8	2.5	5.8	5.1	1.8	4.0	8.5	14.4	18.4	11.8	14.7
L. acidophilus NCTC 1899	3.1	12.4	4.9	21.5	11.8	6.2	0.7	1.5	2.1	4.8	1.9	2.9
L. fermenti ATCC 9338	0	3.2	1.4	5.1	2.9	3.0	0	2.4	2.0	8.4	2.0	3.0
L. lactis ATCC 8000	0.11	2.8	1.5	1.9	3.1	2.2	0.11	4.1	2.2	4.1	3.8	4.1
Str. salivarius	0	0.15	0.09	0.16	0.12	0.05	0	0.11	0.25	0.11	0.10	0.30
Str. pyogenes ATCC 6636	0	1.1	0.09	1.8	1.2	1.5	0	1.5	3.2	1.5	2.8	3.2
Str. pyogenes (*haemolyticus*) ATCC 9342	0	1.1	0.7	3.0	2.2	1.9	0	2.1	1.5	2.1	2.0	2.1
Str. sp. ATCC 9854	0	1.4	0.05	2.1	0.4	1.2	0	1.9	1.9	1.7	1.5	1.6
Str. sp. (*viridans* type) NCTC 3165	0	5.2	2.1	7.6	6.9	5.1	0	1.6	17.5	17.5	21.0	6.8
Corynebacterium bovis NCTC 3224	0.6	4.4	2.5	7.5	6.4	5.3	2.0	10.5	11.2	8.4	8.5	8.5
E. coli 113	0	10.7	14.5	14.5	14.5	4.4	0.09	3.7	4.2	3.4	4.3	2.2
E. coli 154	0	6.9	4.9	12.8	8.0	5.0	0.11	3.2	3.6	3.4	3.6	3.3

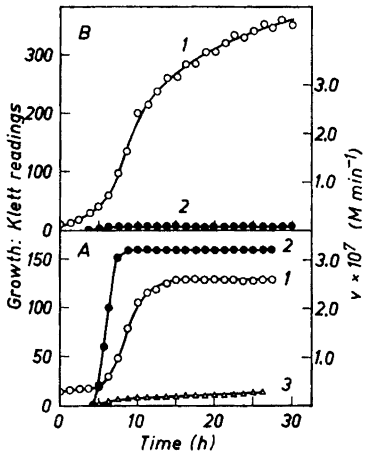

Fig. 14. Formation of enzymes possessing proline iminopeptidase activity in *Lactobacillus casei* NCTC 10302 (A) and *Streptococcus salivarius* (B) cells during the growth. 1: growth curve (lefthand scale); 2: enzyme activity in the cells; 3: enzyme activity in the growth medium.

under other conditions the test organisms used may alter their formation of enzymes possessing proline iminopeptidase activity. Evidently there are also other micro-organisms in the human oral cavity (than the supposed lactobacilli) which may be able to form such enzymes.

Fig. 14 shows experiments in which the biosynthesis of proline iminopeptidase in *L. casei* and *Str. salivarius* was studied. Also this result clearly reveals the differences between lactobacilli and streptococci in their ability to synthesize enzymes possessing proline iminopeptidase activity. It also shows that the formation of the enzyme(s) in *L. casei* started simultaneously with the cell division and that the highest enzyme activity in the cells was not found in any restricted growth phase but rather during the whole life cycle.

DISCUSSION

The enzymes described in the present investigation are in large produced by oral micro-organisms. They may be termed salivary enzymes only in case the material (whole saliva) used in most experiments as source of the proline iminopeptidase activity is considered saliva. In this study such practice has been followed and the material used in many of the presented experiments, the human whole saliva, thus contained also ingredients other than those purely derived from salivary glands. Such extraneous components were epithelial cells, food debris, micro-organisms, *etc.*

A portion of the oral iminopeptidase activity was shown to be due to the involvement of unspecific proteolytic or esterolytic enzymes (like the enzyme termed proline iminopeptidase I in this paper), while a portion was specific as to the nature of the amino acid part of the substrate molecule. That there are both unspecific and more specific enzymes displaying proline iminopeptidase

activity in the human oral cavity, has been already demonstrated in earlier papers.[4-6] The results of the present study showed that human oral cavity indeed contains at least two rather specific proline iminopeptidases, cleaving most rapidly the 2-naphthylamides of L-proline and L-hydroxyproline of altogether 25 different amino acid 2-naphthylamides tested. The substrate specificity studies revealed that at least the more thoroughly purified enzyme did not display endopeptidase activity. Of the three enzymes found that one termed proline iminopeptidase I clearly differed in most enzymatic properties studied from those termed proline iminopeptidases II and III. It is very likely that the human oral cavity contains even further enzymes possessing proline iminopeptidase activity, and which could be identified by modified chromatographic procedures.

The mutual similarity of the plaque and salivary proline iminopeptidases was so striking, as proved by several experiments, that the same enzymes can be said to occur and act in both. It has been demonstrated elsewhere [25,26] that the proline iminopeptidases of even human carious dentine are identical with enzymes occurring in the whole saliva and in the dental plaque. The few micro-organisms selected for the present study were all able to form enzymes displaying proline iminopeptidase activity. It seems to be evident, however, that the oral streptococci are not very effective in the formation of such enzymes in the conditions used in this study. In the present study the eventual contribution of loose epithelial cells or fluids derived from the oral mucous membrane has not been considered.

The significance of the proline iminopeptidases identified in the human oral cavity could be evaluated perhaps on the basis of the substrate specificity of the enzymes involved. As to their importance in relation to oral diseases (caries, periodontal diseases, etc.), this can be determined only after enough information has been gathered about the chemical fine structure of the constituents of the oral tissues involved (see, however, the discussions on this same subject presented elsewhere [4,5,25,26]). While it seems probable that the proline iminopeptidases described act on various compounds derived from food, micro-organisms, *etc.*, their possible direct action on oral tissues remains to be investigated. One should bear in mind, however, that the fairly large number of different enzymes in the bacterial plaque and saliva furnishes them with a wide "total substrate specificity", although several strictly specific enzymes certainly are present. The proline iminopeptidases investigated in this paper form a portion of that "total hydrolytic enzyme activity". It may be concluded that almost any kind of chemical bond generally susceptible to enzymic hydrolysis, is likewise susceptible if it is present in oral hard tissues if the conditions become favourable enough to permit enzyme action.

The use of the various chemical compounds presented in Table 5 showed that *p*-toluenesulphonyl-L-phenylalanylchloromethyl ketone and *N*α-toluenesulphonyl-L-lysyl chloromethane had no strong inhibiting effect on the action of any of the three proline iminopeptidases discovered. The former compound inactivates α-chymotrypsin (EC 3.4.4.5) stoichiometrically through alkylation of a histidine residue.[30] It inactivates also liver aminopeptidase B.[13] Because it fails to act on trypsin (EC 3.4.4.4),[31] it may be thought that the

natural substrate(s) of the proline iminopeptidases studied more closely resembles in structure the substrates of trypsin than those of α-chymotrypsin, and that the proline iminopeptidases and trypsin possess certain similarities in their action. This assumption is not, however, well grounded, because Nα-tosyl-L-lysyl chloromethane inactivates trypsin specifically and irreversibly. Chymotrypsin is not affected in any way.[29]

The rather strong inhibitory effect of *N*-ethylmaleimide in the case of proline iminopeptidase I supports the concept of participation of SH-groups in the action of the enzyme involved. This is indeed very evident because also *p*-chloromercuribenzoate caused an inhibition. The idea is further supported by the observed activation by L-cysteine and dithiothreitol. The other two enzymes clearly differed in this property from the first mentioned. The other tested compounds, EDTA, phenylmethane sulphonylfluoride, and diphenylcarbamylchloride, all displayed similar results with all of the three proline iminopeptidases. As a consequence of the results with EDTA it can be stated that none of them are metalloenzymes. In this way they differ from the proline iminopeptidase of *E. coli*.[1] The low inhibitory effect of phenylmethane sulphonylfluoride will not provide sufficient support for assuming any participation of a serine residue in the action of the enzymes discovered. This compound inhibits α-chymotrypsin.[32] This inhibition evidently results from a reaction with the hydroxyl group of the serine residue at the active site.[32] The most promptly inhibiting compound tested was diphenylcarbamyl chloride. The K_i value of about 6.0×10^{-5} M in the inactivation of α-chymotrypsin[28,29] may be compared with that given in the action of the more thoroughly purified enzyme in the present study. The compound is considered a specific inactivator of α-chymotrypsin, resulting from a 1:1 stoichiometric reaction.[28,33]

The effect of pH and substrate concentration on the rate of the hydrolysis of L-prolyl-2-naphthylamide by the three enzyme preparations revealed that the pattern of these effects was very nearly the same with all of the enzyme preparations. The essentially similar pattern of the effect of pH suggests that the same amino acid side groups could be responsible for the activity of all of the enzymes (this can be said only with certain reservation, because the effect of *N*-ethylmaleimide and *p*-chloromercuribenzoate was different). Any suggestion about a more close nature of the amino acid groups necessary in the action of the proline iminopeptidases cannot be made on the basis of the present results. It can be concluded, however, that the presence in the substrate molecule of the imino ring creates different requirements as regards amino acid residues necessary at the active site from that found for aminopeptidase B, acting specifically on *N*-terminal L-arginyl- and L-lysyl-residues. One difference is the unlikely involvement of a specific histidine residue in the catalyses by the enzymes involved. Therefore, the participation of such a nucleophile as imidazole in any of the three proline iminopeptidases on the basis of the results given cannot be considered. Fig. 11 revealed a further difference in the pattern of the effect of pH in phosphate buffer between liver aminopeptidase and the present enzymes. This is the positive slope of the pH-dependence curves at pH 6.2—7.5. The slope of this upward section of the

of studies on the enzymes involved found in the literature to reveal the considerable variation in the properties of various enzymes. The K_m values and the effects of pH have been determined in different buffers. Therefore, they cannot be compared very strictly with each other. Abbreviations: 2-NA = 2-naphthylamine; pCMB = *p*-chloromercuribenzoate; Me^{2+} = divalent metal cation; — = no data was available.

Source	Substrate(s)	Requirements for metals	Involvement of -SH groups in the catalysis	pH-Optimum	K_m (M)	Ref.
Human saliva and plaque (the present enzymes: I, II, and III)	L-Prolyl-2-NA L-OH-Prolyl-2-NA	Not found	I Obvious II Not found III Not found	I 8.0 II 8.0 III 8.0	I 1.0×10^{-3} II 1.0×10^{-4} III 1.0×10^{-4} (L-Prolyl-2-NA)	This work
Rat liver microsomes (dipeptide naphthylamidase)	Glycyl-L-Prolyl-2-NA	Not found	Not found	7.8	1.56×10^{-4}	35
Neisseria catarrhalis	L-Alanyl-2-NA L-Leucyl-2-NA *etc.*	Not found	Obvious	7.3	8.0×10^{-5} (L-Alanyl-2-NA) 1.2×10^{-5} (L-Leucyl-2-NA)	36
Mice endocrine pancreas	L-Leucyl-2-NA	—	—	7.0	4.0×10^{-5} 6.0×10^{-5}	37
Rat liver and kidney (lysosomes and microsomes)	L-Alanyl-2-NA L-Arginyl-2-NA	Microsomal enzyme is act. by Co^{2+}. Lysosomal enzymes are inhibited by certain Me^{2+} ions	pCMB inhibits the lysosomal enzyme of liver, not that of kidney microsomes	7.0—7.5	—	38
E. coli (proline iminopeptidase)	Poly-L-proline	Mn^{2+} activates	Obvious	7.8—9.5	—	1
Aeromonas proteolytica	L-Leucyl-2-NA	Zn^{2+} activates	—	8 —9		39
Bovine anterior pituitary	L-Arginyl-2-NA L-Lysyl-2-NA	Some divalent metal cations activate	Obvious	7.0		40
Man: liver and duodenal mucosa (two isoenzymes?)	L-Alanyl-2-NA	—	—	Double pH optimum (?)	1.0×10^{-3} (at pH 9)	41
Man, cat, rat, and guinea pig liver (aminopeptidase B)	L-Arginyl-2-NA L-Lysyl-2-NA	Not found	Obvious	7.0	1.0×10^{-4}	9, 10, 11, 12

curve was approximately 0.5 units (similar values can be obtained with the experimental points of Fig. 10, if log v is plotted against pH) whereas the corresponding slope with liver aminopeptidase B in phosphate buffer has been found to be considerably lower.[12] When the slope of the pK_m *versus* pH curves obtained with a dipeptide naphthylamidase (liberating glycyl-DL-proline from glycyl-DL-prolyl-2-naphthylamide)[34] is calculated, the following values are obtained: approximately 1.3 for both the acidic and basic linear sections of the curve, in 0.1 M barbiturate buffer. This value is somewhat higher than the usually expected value (one-unit slope), in cases where the mechanism of Alberty and Massey[41] can be applied. This mechanism is not applicable without certain alterations for the three proline iminopeptidases or aminopeptidase B. With these latter enzymes a modified reaction scheme can be expected, for example, one in which the protonated form of enzyme substrate complex (ES^{n+1}) breaks down to form E^n and products (in addition of the breakdown *via* ES^n), particularly, when the acid side of the pH-dependence curves is considered. The experiments documented in Fig. 10 yielded curves with a clearly detectable linear section with zero slope. Hence the pK values of the ionizing groups involved are located far enough from each other to permit a graphical determination of the value of these constants. The observed pattern of the effect of pH on the proline iminopeptidases has been detected also in some earlier studies with more crude enzyme preparations.[4,5,26] An additional difference between aminopeptidase B and the proline iminopeptidases is in the effect of NaCl: aminopeptidase B is strongly activated by sodium chloride,[10,12] whereas the three proline iminopeptidases are not. When compared with other arylaminopeptidases (or iminopeptidases) several differences can be observed as is shown in Table 7. Unfortunately, most arylaminopeptidases are inadequately purified to permit a closer comparison between the different arylaminopeptidase-like enzymes. It is, however, evident, that in despite of the close similarity in the structure, the various amino acid 2-naphthylamides are hydrolyzed in enzymic catalyses of very varying nature.

Finally, it is to be pointed out that it is very much possible that no one of the three proline iminopeptidases described are the same as those formed by the cells of *L. casei* NCTC 10302. This assumption receives some support from observations made on the preservation of various proline iminopeptidase-like enzymes in polyacrylamide columns. The activity of the oral proline iminopeptidases of this study was rather easily demonstrated in the gel slabs. However, in certain experiments not described here in more detail,[42] the proline iminopeptidase activity possessing enzyme of *L. casei* NCTC 10302, grown in TSHGA medium, has been found in this laboratory to undergo a process in the polyacrylamide columns, leading to irreversible inactivation of the enzyme. In the same experiments it was also found that this *L. casei* enzyme displays a wider substrate specificity: the 2-naphthylamides of several L-amino acids are obviously hydrolyzed. Consequently, the source of the oral proline iminopeptidases may be (also) other than the cells of *L. casei*.

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