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DIPEPTIDYL PEPTIDASE II OF BOVINE DENTAL PULP

INITIAL DEMONSTRATION AND CHARACTERIZATION AS A FIBROBLASTIC, LYSOSOMAL PEPTIDASE OF THE SERINE CLASS ACTIVE ON COLLAGEN-RELATED PEPTIDES

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Summary

Dipeptidyl peptidase II (dipeptidylpeptide hydrolase, EC 3.4.14.2), previously known as dipeptidyl aminopeptidase II, was shown to be present in relatively high concentrations in bovine dental pulp. Based on the DNA content of tissue homogenates, the fibroblasts of this connective tissue appeared to contain more dipeptidyl peptidase II than the cells of lysosome-rich tissues such as bovine spleen and rat liver.

The newly-recognized properties of dipeptidyl peptidase II, from both pulp and pituitary sources, included a marked propensity for hydrolyzing prolyl bonds at acidic pH. Lys-Pro-2-NNap and Lys-Pro-2(4-methoxy)naphthylamide (designed for cytochemical application) were hydrolyzed at rates equal to that of Lys-Ala-2-NNap. The impure pulp enzyme and the authentic pituitary enzyme showed comparable relative rates of hydrolysis on a variety of fluorogenic substrates with the general structure X-Pro-2-NNap (X = Lys, Arg, Phe, Ala or Gly), and on a variety of collagen-related tripeptides represented by X-Pro-Ala (X = Gly, Ala or Lys). The highest rates were obtained on Lys-Ala-Ala and Lys-Ala-Pro. The pH optima for the hydrolysis of the 2-naphthylamide derivatives varied from 5.0 to 5.7, and for tripeptides from 4.2 to 5.3. In all cases the N-terminal dipeptide was released intact. Although previously un-

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Abbreviations: 2-NNap, 2-naphthylamide; NNapODe, 2-(4-methoxy)naphthylamide; *t*-Boc, *t*-butyloxycarbonyl; Cbz, benzyloxycarbonyl; Dip-F, diisopropyl phosphorofluoridate; CH_2Cl, chloromethyl ketone; Tos, *p*-tolylsulphonyl; Bz, benzoyl; Ac, *N*-acetyl.

recognized as a serine protease, dipeptidyl peptidase II (of pulp and pituitary origin) was strongly inhibited by (1 mM) diisopropyl phosphorofluoridate, *p*-nitrophenyl-*p'*-guanidinobenzoate, and phenylmethylsulfonyl fluoride. The enzyme, from both sources, was fully inhibited by 0.1 mM Lys-Ala chloromethyl ketone, a newly-designed, active-site-directed inhibitor.

The numerous properties shared by the putative dipeptidyl peptidase II of bovine dental pulp and an authentic preparation of the bovine pituitary enzyme provided strong support for their having a common identity.

Introduction

Dipeptidyl peptidase II (dipeptidylpeptide hydrolase, EC 3.4.14.2), originally named dipeptidyl aminopeptidase II, was first recognized as one of a family of three such enzymes present in aqueous extracts of bovine pituitary glands [1,2]. All members of this class of dipeptidylpeptide hydrolases (EC 3.4.14.—) characteristically catalyze the successive release of dipeptide moieties from the N-termini of polypeptides. To date, four such enzymes have been purified and characterized. Their substrate specificities, subcellular localization and tissue distribution are summarized elsewhere [3]. Although dipeptidyl peptidase I and II are both lysosomal enzymes active at acidic pH, only dipeptidyl peptidase II catalyzes the hydrolysis of Lys-Ala-2-NNap to release intact lysyl-alanine. Many other dipeptidyl-2-NNap substrates have been tested, but Lys-Ala-2-NNap has, until now, served as the most sensitive and specific fluorogenic substrate for the assay of dipeptidyl peptidase II. Similarly, Lys-Ala-NNapOMe has been used as a reliable marker for the visualization of lysosomal structures by electron microscopy [3,4]. As noted in this report, the corresponding derivatives of Lys-Pro may prove to be even more useful.

As part of an attempt to identify the lysosomal enzymes responsible for the observed intracellular (lysosomal) degradation of collagen by fibroblasts [5], the present investigation represents an initial study of a putative dipeptidyl peptidase II that was recently found to be abundant in bovine dental pulp, a connective tissue that consists predominantly of fibroblasts imbedded in a collagen-proteoglycan matrix [6]. Because dipeptidyl peptidase II purified over 1000-fold from bovine pituitaries was previously reported [2] to exhibit a measureable rate of hydrolysis on Gly-Pro-2-NNap at pH 5.5, the prolyl-bound cleaving activities of both the impure pulp enzyme and the authentic (pituitary) enzyme were characterized and compared, as were their sensitivities to a variety of inhibitors, especially those acting on serine proteases.

Materials and Methods

The peptides and peptidyl-2-naphthylamide derivatives used in this study were obtained from Bachem, Inc. (Torrance, CA). Those that were custom synthesized by Bachem included all of the proline-containing peptides, Lys-Ala-Ala, and Lys-Ala chloromethyl ketone. The 2-(4-methoxy)naphthylamide derivative of Lys-Pro was custom synthesized by Enzyme Systems Products (Indianapolis, IN). The purity and identity of the peptides were confirmed by

TLC and amino acid analysis. Those found to contain impurities were subjected to preparative TLC on 1 mm-thick layers of Avicel microcrystalline cellulose purchased, as precoated glass plates (20 × 20 cm), from Analtech, Inc., (Newark, DE). The plates were developed with *n*-butanol/formic acid/water (70 : 15 : 15) and the peptides recovered by extracting the cellulose with dilute HCl. Unless indicated otherwise, all peptides were comprised of residues having the L configuration.

Diisopropyl phosphorofluoridate was purchased from Calbiochem (San Diego, CA) and *p*-nitrophenyl-*p'*-guanidinobenzoate from ICN Pharmaceuticals, Inc. (Cleveland, OH). Hyamine 10-X (diisobutylcresoxyethoxyethyl dimethyl benzyl ammonium chloride) was obtained from Rohm and Haas (Philadelphia, PA). All other biochemicals used in this study were purchased from Sigma Chemical Company (Saint Louis, MO).

The fluorimetric assay of dipitidyl peptidase II. The activity of dipeptidyl peptidase II was assayed on dipeptidyl-2-naphthylamide substrates (the standard assay substrate being L-Lys-L-Ala-2NNap) at pH 5.5, by adding 0.1 ml enzyme to 3.9 ml assay solution (at 37°C) comprised of 1.9 ml water/1 ml 0.25 M cacodylic acid-NaOH buffer, pH 5.5/1 ml 0.8 mM Lys-Ala-2-NNap (0.2 mM final). As described in detail elsewhere [7], reaction rates were measured by a direct, recording technique using a Turner fluorometer (Model 111) calibrated with free 2-naphthylamine and with 2-(4-methoxy)naphthylamine. 1 unit of dipeptidyl peptidase II activity was the amount of enzyme that catalyzed the release of 1 μmol 2-naphthylamine from Lys-Ala-2-NNap per min at pH 5.5 and 37°C under the above assay conditions.

Preparation and fractionation of pulp extracts. Bovine dental pulp was collected at a local abattoir from the unerupted distal molars of freshly-killed heifers. Each unerupted molar yielded 3—4 g connective tissue that was transported on ice to the laboratory. The pulps were combined with four parts (by weight) cold water and completely dispersed with a PT-20 Polytron homogenizer (Brinkmann Instruments) to yield the '20% homogenate'. A '20% aqueous extract' was obtained by centrifuging the homogenate at 15 000 × *g* for 30 min at 4°C. Aliquots were stored at —70°C.

An enriched, stable, working preparation of freeze-dried enzyme was obtained as follows. Frozen, bovine dental pulp was obtained in quantity from the Wilson Packing Company (Cedar Rapids, IA) and stored at —70°C. Whole pulp tissue was subsequently freeze-dried, pulverized in a Waring blender and ground to a 60-mesh powder in a bench-type Wiley mill operated at room temperature with frequent pauses to avoid heating. The pulp powder was stored over desiccant at —20°C. A subsequent enrichment step was carried out by suspending 100 g of the pulp powder in 1 l cold 5 mM Tris-HCl buffer, pH 7.8, and stirring for 1 h. This and subsequent steps were conducted at 4°C. The suspension was centrifuged at 12 000 × *g* for 30 min and about 700 ml supernatant recovered. The pellet was extracted a second time into 700 ml Tris-HCl buffer by stirring for 30 min and centrifuging as before. The two supernatants were combined to give about 1400 ml aqueous extract containing 37 mg dry weight/ml and having a specific activity of 5 munits per mg dry weight. Solid $(NH_4)_2SO_4$ was stirred into the cold aqueous extract until the mixture was 40% saturated. The protein precipitated between 0 and 40% saturated

$(NH_4)_2SO_4$, designated the '$(NH_4)_2SO_4$ fraction', was recovered by centrifugation, dialyzed against water until free of sulfate and finally freeze-dried. This was a stable source of dipeptidyl peptidase II that contained 30 munits of activity/mg dry weight.

Preparation of a reference (pituitary) sample of dipeptidyl peptidase II. Because dipeptidyl peptidase II was originally detected in bovine pituitary extracts [1] and was characterized using a preparation purified 1000-fold from this source [2], an enriched preparation of the pituitary enzyme was therefore used as a standard for the comparative studies described in this report. This reference consisted of the IRC-50 column fraction from the purification scheme previously described [2], which was approx. 700-fold purified over the pH 5.5 aqueous pituitary extract. The published results of electrophoresis at pH 5.0 in polyacrylamide gel showed dipeptidyl peptidase II to be the major component in the IRC-50 fraction; however, four other contaminating proteins were detected. The dipeptidyl peptidase II in the IRC-50 fraction had a specific activity of approx. 12 units/mg protein when freshly-prepared. The IRC-50 fraction used as a reference in the present study was a freeze-dried aliquot that had lost some activity in storage and had a specific activity of about 3 units/mg protein when used in the present study.

pH studies with peptidyl-2-naphthylamide substrates. For each point on the pH curves, a buffered substrate solution was prepared by combining 1.0 ml 0.8 mM substrate, 1.0 ml 0.2 M cacodylic acid-NaOH buffer (appropriate pH), and 1.9 ml water. These substrate solutions were brought to 37°C and (at 1-min intervals) each reaction was initiated by adding 0.1 ml of a 20% (w/v) whole homogenate to give a final volume of 4 ml. The final pH was measured and at each reaction time (10, 20 and 30 min) a 0.4-ml aliquot was combined with 3.6 ml 50 mM Tris-HCl buffer, pH 9.0, to stop the reaction and circumvent the need to correct for the acid quenching of fluorescence that occurs below pH 5.0. 2-Naphthylamine concentrations were measured fluorimetrically as described above. Amounts of 2-naphthylamine liberated at each time interval were used to calculate rates of hydrolysis.

Measurement of the hydrolysis rates on tripeptides. Reaction mixtures were prepared by combining 100 μl 0.2 M cacodylic acid-NaOH buffer, pH 5.0, with 100 μl (0.2—2.0 mg) of the '$(NH_4)_2SO_4$ fraction' in water. The mixture was preincubated 2 min at 37°C and the reaction initiated by adding 200 μl of a 20 mM aqueous solution of tripeptide prewarmed to 37°C. Since many of the tripeptides were hygroscopic, concentrations of stock solutions were established by amino acid analysis and by quantitation of liberated amino acids following complete enzymatic hydrolysis. At specified time intervals (usually 10, 20, and 30 min), 15-μl aliquots were mixed with 75 μl cold 0.2 M sodium citrate buffer, pH 2.2, and stored frozen until analyzed. The identity and quantity of the liberated, C-terminal amino acid was established on a Durrum D-502 amino acid analyzer by applying 20 μl quantities (equivalent to 33 nmol tripeptide) of the stored aliquots. Rates of tripeptide hydrolysis were linear up to about 50% hydrolysis and all rates were established within this interval. Tripeptide fragments were separated on the analyzer using standard operating conditions involving the use of sodium citrate buffers at pH 3.25, 4.25 and 7.9.

pH studies with tripeptide substrates. For each point on the pH curves, a

buffered enzyme solution was prepared by combining 100 μl 0.2 M cacodylic acid-NaOH buffer (appropriate pH) and 100 μl enzyme solution that contained 0.2—2.0 mg of the '$(NH_4)_2SO_4$ fraction' in water. The enzyme solutions were brought to 37°C and (at 1-min intervals) each reaction was initiated by adding 200 μl 20 mM tripeptide in water at 37°C. The tripeptide solutions were previously adjusted to approx. pH 3.5, 5, or 6 for use in the low, middle, or high range of the pH curve. After 10, 20, and 30 min of incubation, a 15-μl aliquot of each reaction was combined with 75 μl cold 0.2 M sodium citrate buffer, pH 2.2. These mixtures were stored frozen until 20-μl aliquots (equivalent to 33 nmol substrate) were taken for amino acid analysis as described above.

Time course analysis of tripeptide digests by TLC. Reactions were initiated by combining 200 μl 20 mM tripeptide, pH 5.0, with 200 μl of a dipeptidyl peptidase II solution containing 2 mg of the '$(NH_4)_2SO_4$ fraction' in 0.1 M cacodylic acid-NaOH buffer, pH 5.0. Immediately thereafter, a 1-μl aliquot was spotted at 'zero time' and at intervals of 10, 20, 30, 45, 60 and 90 min. The reaction was maintained at 37°C. Standards were applied in 3 nmol quantities. The plate was developed with 80% aqueous phenol and allowed to air dry overnight. The peptides were detected with a ninhydrin reagent that contained 50 ml 0.2% ninhydrin in absolute ethanol, 10 ml glacial acetic acid and 2 ml 2,4,6-collidine. The plates were heated at 110°C for 2 min.

Other assay methods. Dipeptidyl peptidase I (dipeptidyl aminopeptidase I, cathepsin C) was assayed fluorimetrically on Gly-Phe-2-NNap at pH 6.0 using 2-mercaptoethylamine hydrochloride to satisfy the enzyme's sulfhydryl and halide requirements [7]. Cathepsin B (previously designated cathepsin B1) was assayed fluorimetrically on Cbz-Arg-Arg-2-NNap at pH 6.5 in the presence of dithiothreitol and EDTA. As previously reported [8], this substrate provides a 70-fold greater sensitivity compared to Bz-DL-Arg-2-NNap for the assay of cathepsin B. For the fluorimetric assays, 1 unit enzyme was the amount that catalyzed the release of 1 μmol of 2-naphthylamine per min under the conditions of the assay. Cathepsin D was assayed on hemoglobin at pH 4 as described by Schwabe [9] wherein the TCA-soluble products are quantitated with the Folin-Ciocalteau reagent. 1 unit cathepsin D was taken to be the amount of enzyme that produced an absorbance increase of 0.1 at 700 nm ($\Delta A_{700}^{1cm} = 0.1$) per h under the conditions of the assay. The DNA content of tissue homogenates was estimated by the diphenylamine method of Burton [10] as modified by Giles and Myers [11] to increase sensitivity, and with color development at reduced temperature as recommended by Croft and Lubran [12] to exclude the color contribution of sialic acid; it has been reported by Orlowski [13] that bovine dental pulp contains a relatively high concentration of sialic acid. Protein was determined by the method of Lowry et al. [14] employing bovine serum albumin as the standard.

Results

Effect of pH on the rate of hydrolysis of dipeptidyl-2-naphthylamide substrates by a pulp homogenate. The maximum rate of hydrolysis of Lys-Ala-2-NNap by a whole homogenate of fresh pulp occurred at pH 5.5. This pH optimum, shown in Fig. 1, is identical to that reported earlier [2] for the purified

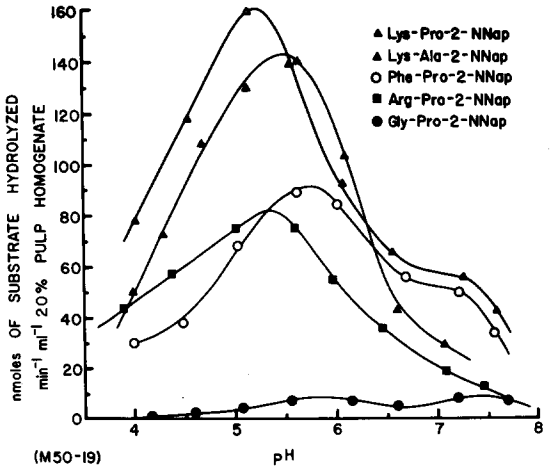

Fig. 1. Effect of pH on the rate of hydrolysis of various dipeptidyl-2-naphthylamides by a whole homogenate of bovine dental pulp.

pituitary dipeptidyl peptidase II. The rates of hydrolysis of the prolyl bonds in Lys-Pro-2-NNap, Arg-Pro-2-NNap and Phe-Pro-2-NNap were optimal at about pH 5.2, 5.3 and 5.7, respectively. At pH 5.5 the rates on Lys-Ala-2-NNap and Lys-Pro-2-NNap were essentially equal. The rate of hydrolysis on Gly-Pro-2-NNap, which was relatively low compared to the other substrates, appeared to be maximal between pH 5.5 and 6.0. Elevated rates of hydrolysis on all the proline-containing substrates were also detected between pH 7 and 8, but these activities were, most probably, attributable to dipeptidyl peptidase IV since this enzyme has been detected in bovine dental pulp [15] and is known to hydrolyze substrates of this type in this pH range [16]. For subsequent studies, pH 5.5 was selected as a reasonable compromise for all dipeptidyl peptidase II assays conducted on dipeptidyl-2-naphthylamide substrates. Although Ala-2-NNap was readily hydrolyzed by the pulp homogenate at pH 7.0, no activity was detected on this substrate, nor on Pro-2-NNap, at pH 5.5; consequently, the rates of hydrolysis of the dipeptide derivatives measured at pH 5.5 were not complicated by the stepwise degradation of the substrates by (aminoacyl) aminopeptidases.

Comparison of the substrate specificities of pulp and pituitary dipeptidyl peptidase II activities on a range of dipeptidyl-2-naphthylamide substrates. The substrate specificities for both the putative dipeptidyl peptidase II contained in the '$(NH_4)_2SO_4$ fraction' from bovine dental pulp, and the reference pituitary dipeptidyl peptidase II were compared on a range of dipeptidyl-2-naphthylamide substrates. As illustrated in Table I, both the pulp and pituitary preparations exhibited maximum rates of hydrolysis on Lys-Ala-2-NNap that were followed by near-identical relative rates on a variety of substrates that included those containing a penultimate prolyl residue, i.e., Lys-Pro-2-NNap, Lys-Pro-NNapOMe, Phe-Pro-2-NNap and Arg-Pro-2-NNap. Although the absolute rate of hydrolysis of the NNapOMe derivative of Lys-Pro was no greater than that of the 2-NNap derivative, the former did provide a 2-fold increase in sensitivity that was attributable to the greater fluorescence yield from 2-(4-methyoxy)-

TABLE I

COMPARISON OF THE RELATIVE RATES OF HYDROLYSIS AT pH 5.5 OF A VARIETY OF DIPEPTIDYL-2-NAPHTHYLAMIDE SUBSTRATES BY A PUTATIVE DIPEPTIDYL PEPTIDASE II FROM BOVINE DENTAL PULP AND AN AUTHENTIC PREPARATION OF THE BOVINE PITUITARY ENZYME

The source of the putative dipeptidyl peptidase II activity of bovine dental pulp was the '$(NH_4)_2SO_4$ fraction'. The specific activity on Lys-Ala-2-NNap was 0.3 unit/mg dry weight. The reference sample of bovine pituitary dipeptidyl peptidase II was the 'IRC-50 fraction'. The specific activity on Lys-Ala-2-NNap was 2.9 units/mg protein.

Substrate	Source of dipeptidyl peptidase II activity	
	Bovine pulp (%)	Bovine pituitary (%)
Lys-Ala-2-NNap	100	100
Lys-Pro-2-NNap	100	100
Lys-Pro-NNapOMe	100	100
N^{α},N^{ϵ}-Cbz_2-Lys-Ala-2-NNap	0	0
Phe-Pro-2-NNap	67	75
Arg-Pro-2NNap	63	62
t-Boc-Arg-Pro-2-NNap	0	0
Ala-Pro-2-NNap	45	58
Arg-Ala-2-NNap	27	30
Leu-Ala-2-NNap	14	10
Ala-Ala-2-NNap	6	7
Gly-Pro-2-NNap	3	5
Gly-Phe-2-NNap	0	0
Gly-Arg-2-NNap	0	0
Arg-Arg-2-NNap	0	0
Ala-2-NNap	0	0
Pro-2-NNap	0	0

naphthylamine. When pulp extracts were first found to hydrolyze these proline-containing substrates at acidic pH, dipeptidyl peptidase II was not immediately suspected as the responsible enzyme; however, an examination of the pituitary enzyme on these substrates revealed that dipeptidyl peptidase II was indeed capable of catalyzing the rapid hydrolysis of prolyl bonds. Furthermore, the lack of activity by both the pulp and pituitary preparations on *t*-Boc-Arg-Pro-2-NNap and Cbz-Lys-Ala-2-NNap demonstrated that an unsubstituted α-amino group was essential for the removal of the intact dipeptide. The rates at which dipeptidyl peptidase II hydrolyzed the dipeptidyl-2-naphthylamides appeared to be greatly influenced by the size, rather than the class, of the N-terminal residue of the substrate. For example, significantly greater rates of hydrolysis were observed when N-terminal glycine was replaced by alanine and still greater rates when the N-terminal residue was replaced by arginine, phenylalanine, or lysine. Proline and alanine seemed to be the preferred penultimate residues. The identification of the split products by TLC provided direct evidence that the action of both the pulp and pituitary preparations on the dipeptide derivatives resulted in the release of intact dipeptides. Substrates used for the assay of dipeptidyl peptidase I (Gly-Phe-2-NNap and Gly-Arg-2-NNap) and dipeptidyl peptidase III (Arg-Arg-2-NNap) were not hydrolyzed.

Effect of pH on the rate of tripeptide hydrolysis by an enriched pulp fraction. The pH curves shown in Fig. 2 reveal that the '$(NH_4)_2SO_4$ fraction' catalyzed the hydrolysis of a range of tripeptide substrates most rapidly between

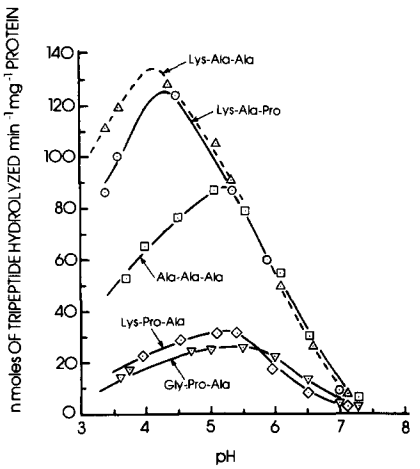

Fig. 2. Effect of pH on the rate of hydrolysis of various tripeptides by an enriched dipeptidyl peptidase II fraction derived from bovine dental pulp by $(NH_4)_2SO_4$ fractionation.

pH 4.0 and 5.5. The absolute rate of hydrolysis of Lys-Ala-Ala by pulp dipeptidyl peptidase II at pH 4.2 (optimum) was about 4-times greater than the rate on Lys-Ala-2-NNap (the standard assay substrate) at its pH 5.5 optimum. Tripeptides with penultimate prolyl residues were hydrolyzed best betweeen pH 5.0 and 5.5. In general, the pH optima exhibited on tripeptides were about 0.5—1.5 units lower than those observed on the dipeptidyl-2-naphthylamides (Fig. 1).

Relative rates and split products of tripeptide hydrolysis at pH 5.0. The rates of hydrolysis on a range of tripeptide substrates were compared at pH 5.0 using the '$(NH_4)_2SO_4$ fraction' from bovine pulp. The relative rates of dipeptide removal, measured as the rate of release of the C-terminal residue (as described under Materials and Methods) were compared to rates similarly measured using the reference preparation of bovine pituitary dipeptidyl peptidase II. The relative rates of hydrolysis exhibited by the two preparations on six different tripeptides were virtually identical. Rates on Lys-Ala-Ala and Lys-Ala-Pro were the highest (100%), followed by Ala-Ala-Ala (80%), Lys-Pro-Ala (35%), Gly-Pro-Ala (29%) and Ala-Pro-Ala (25%). These results showed that the responsible activity present in the '$(NH_4)_2SO_4$ fraction' (from pulp) was most probably a typical dipeptidyl peptidase II. As was the case for the 2-naphthylamide derivatives, tripeptide substrates containing N-terminal Lys-Ala were hydrolyzed most rapidly. Of relative uniqueness was the ability of dipeptidyl peptidase II to cleave the Ala-Ala linkage (in Lys-Ala-Ala) and the Ala-Pro linkage (in Lys-Ala-Pro) at similar rates, and to cleave, also at substantial rates, Pro-Ala bonds, as for example in Gly-Pro-Ala, a typical 'collagen triplet'. A time course analysis by TLC of the hydrolysis of this collagen-related peptide at pH 5.0 by the pulp '$(NH_4)_2SO_4$ fraction' is shown in Fig. 3. Gly-Pro and alanine were the only split products detected. No free glycine was formed, even in prolonged digests. A similar lack of liberated N-terminal residues was noted on the amino acid analyzer when rate studies were conducted with the various tripeptides. A totally unambiguous determination of the site of cleavage was not attainable by

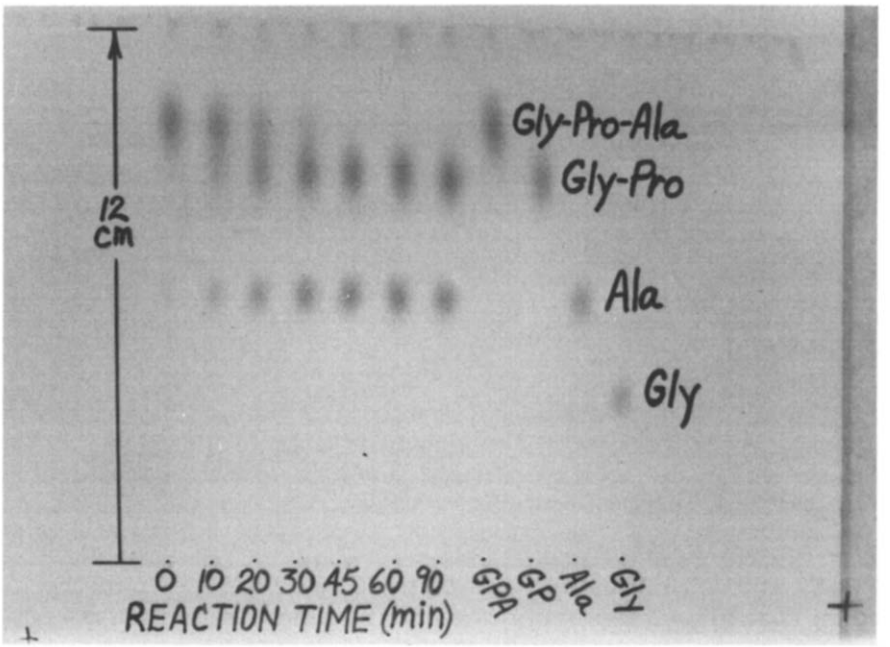

Fig. 3. Time course analysis by TLC of reaction products resulting from the hydrolysis at pH 5.0 of Gly-Pro-Ala by an enriched preparation of dipeptidyl peptidase II from bovine dental pulp.

this procedure for tripeptides having alanine residues at both the N and C-terminals. The lack of action by the pulp fraction on *N*-acetyl-Ala-Ala-Ala was characteristic of the aminopeptidase character of purified pituitary dipeptidyl peptidase II [2].

Effects of potential inhibitors on the putative dipeptidyl peptidase II of dental pulp. Table II compares the effects of various agents on the dipeptidyl peptidase II activity contained in the '$(NH_4)_2SO_4$ fraction' from bovine dental pulp with their effects on the reference preparation of bovine pituitary dipeptidyl peptidase II. The overall similarity of the responses shown by the two preparations provided further justification for considering the responsible enzyme in pulp to be a typical dipeptidyl peptidase II. Strong inhibition by Dip-F and *p*-nitrophenyl-*p'*-guanidinobenzoate, together with substantial inhibition by phenylmethanesulfonyl fluoride, served as the basis for classifying dipeptidyl peptidase II as a serine protease. The effect of serine protease inhibitors on pituitary dipeptidyl peptidase II had not been examined previously. When inhibition (dose-response) curves were compared, the pulp and pituitary preparations were found to be equally sensitive to Dip-F. Although Lys-Ala-2-NNap was used as the assay substrate in the dose-response studies, treatment with Dip-F also eliminated activity against other dipeptidyl-2-naphthylamides (i.e., Lys-Pro-2-NNap and Arg-Pro-2-NNap) and tripeptides (Lys-Ala-Ala, Ala-Pro-Ala, and Ala-Ala-Ala).

The lack of appreciable inhibition by sulfhydryl and chelating reagents spoke for a lack of any active-site requirements for sulfhydryl groups or metal ions. The notable inhibition obtained with $HgCl_2$ was indicative of a heavy metal

TABLE II

COMPARISON OF THE EFFECTS OF POTENTIAL INHIBITORS ON BOVINE PULP AND PITUITARY DIPEPTIDYL PEPTIDASE II

Reactions rates were determined fluorimetrically at pH 5.5, following the addition of Lys-Ala-2-NNap to enzyme/buffer mixtures that had been preincubated with inhibitor for 30 min at 37°C.

Compound	Final concn. (mM)	Inhibition (%)	
		Pulp	Pituitary
Diisopropyl phosphorofluoridate	1.0	90	83
Phenylmethylsulfonyl fluoride	1.0	60	57
p-Nitrophenyl-*p'*-guanidinobenzoate	1.0	100	100
	0.1	70	78
p-Chloromercuriphenyl sulfonate	0.1	23	29
	0.01	0	4
Iodoacetate	1.0	0	0
EDTA	1.0	4	14
$HgCl_2$	0.1	100	93
	0.01	48	67
Lys-AlaCH_2Cl	0.1	100	100
	0.01	72	70
Tos-LysCH_2Cl	0.1	15	27
Tos-PheCH_2Cl	0.1	4	3
Ac-$(Ala)_3$-AlaCH_2Cl	0.1	3	12
NaCl	100	49	52
Tris (at pH 5.5)	50	72	83
Puromycin	1.0	56	72
Hyamine 10-X	0.5	100	97
	0.05	39	44

sensitivity for dipeptidyl peptidase II. The most pronounced inhibition obtained with the chloromethyl ketone derivatives was observed for Lys-AlaCH_2Cl, a new active-site-directed inhibitor that was designed to resemble the assay substrate for dipeptidyl peptidase II. Other chloromethyl ketones, such as those active against trypsin, chymotrypsin, and elastase, (e.g., Tos-LysCH_2Cl, Tos-PheCH_2Cl, and *N*-acetyl-$(Ala)_3$-AlaCH_2Cl) were relatively ineffective. Consistent with the previously-reported sensitivity of pituitary dipeptidyl peptidase II to cations [1], the pulp enzyme was similarly inhibited (at pH 5.5) by sodium, Tris, puromycin, and Hyamine 10-X. Typically, the degree of inhibition increased as the size of the cation increased.

Comparison of bovine dental pulp with two lysosome-rich tissues for their relative content of dipeptidyl peptidase II and three other lysosomal proteases. Aqueous homogenates (20%, w/w) of bovine pulp and bovine spleen, and a 10% (w/w) whole homogenate of rat liver were prepared using a PT-20 Polytron homogenizer in a 4°C cold room. In Table III the activities of dipeptidyl peptidases I and II, cathepsin B and cathepsin D were compared in these three tissues. The comparison was based on the DNA content of the tissues in order to compensate for the collagen and proteoglycan content of the pulp, thereby providing a comparison that more closely reflected the relative amounts of these lysosomal enzymes in the cells comprising these tissues. Typical of other connective tissues, bovine dental pulp contains fibroblasts as the predominant cell type [6]. As seen in Table III, these cells were relatively rich in dipeptidyl

TABLE III

COMPARISON OF THE CELLULAR LEVELS OF FOUR LYSOSOMAL PROTEASE ACTIVITIES IN THREE DIFFERENT TISSUES (munits activity/mg tissue DNA)

mg DNA per g tissue; pulp 1.0, liver 3.5, spleen 10.

Assayed	Tissue surveyed		
	Bovine pulp	Rat liver	Bovine spleen
Dipeptidyl peptidase I	10	925	41
Dipeptidyl peptidase II	700	220	118
Cathepsin B	25	771	1630
Cathepsin D	13	21	18

peptidase II compared to the three other lysosomal proteases assayed in pulp, e.g., dipeptidyl peptidase I, cathepsin B and cathepsin D. In comparison with the cells of rat liver and bovine spleen, both of which are lysosome-rich tissues commonly used as sources of lysosomal enzymes, bovine dental pulp served as a rich source of dipeptidyl peptidase II that lacked the high background of certain lysosomal proteases. This circumstance is expected to facilitate the purification process and yield an enzyme for characterization that is specifically of fibroblastic origin.

Discussion

When we first observed that extracts of bovine dental pulp catalyzed the release of Lys-Ala from Lys-Ala-2-NNap and Lys-Ala-Ala at acidic pH, these activities were tentatively attributed to a connective tissue form of dipeptidyl peptidase II. It was subsequently noted, however, that the pulp extracts were equally effective in releasing Lys-Pro from related proline-containing derivatives, i.e., Lys-Pro-2-NNap and Lys-Pro-Ala. It did appear that dipeptidyl peptidase II might also be responsible for this activity, but there was little justification for making such an assumption since the original studies [1,2] characterizing dipeptidyl peptidase II (using a preparation purified 1000-fold from bovine pituitary glands) had not fully explored its activity on proline-containing substrates. However, when the hydrolytic activities of the pulp extract and purified pituitary dipeptidyl peptidase II were compared on a wide variety of dipeptidyl-2-naphthylamides and tripeptides, that included proline-containing substrates, the two preparations were found to exhibit virtually identical substrate specificities together with common pH optima that varied from pH 4.2 to 5.7; the more acidic conditions usually favored tripeptide hydrolysis.

Both the pituitary and pulpal preparations hydrolyzed Gly-Pro-Ala, a triplet that occurs frequently in collagen, at a much greater rate than was anticipated based on the rate of Gly-Pro-2-NNap hydrolysis, e.g., the rate observed on the tripeptide was about 25-fold faster. There seemed to be a lack of predictability for other rate relationships as well. For example, the rate on Ala-Ala-Ala was as much as 50-fold greater than the rate on Ala-Ala-2-NNap; the rates on Lys-Ala-Ala and Lys-Ala-Pro were only about 5-fold greater than the rate on Lys-Ala-2-NNap; and the rate on Lys-Pro-Ala was about the same as the rate on Lys-Pro-

2-NNap. All rates were compared at their respective pH optima using the data presented in Table I and Fig. 2.

The marked sensitivity of both the pulpal and pituitary activities to serine protease inhibitors was first noted using the pulp extracts; however, a reexamination of pituitary dipeptidyl peptidase II showed that the reference preparation was also a serine protease. The two preparations behaved similarly in studies that compared their sensitivities to Dip-F, phenylmethylsulfonyl fluoride, and *p*-nitrophenyl-*p'*-guanidinobenzoate. Although Chase and Shaw [17] developed *p*-nitrophenyl-*p'*-guanidinobenzoate as a spectrophotometric titrant for trypsin, they reported that other serine proteases, i.e., chymotrypsin, were also reactive. The strong inhibition of dipeptidyl peptidase II suggested that this lysosomal exopeptidase may also be amenable to titration with *p*-nitrophenyl-*p'*-guanidinobenzoate. Both the pulp and pituitary activities exhibited a characteristic sensitivity to cations that was typically greatest for large-molecular-weight cations (Tris, puromycin and Hyamine 10-X). Lys-AlaCH_2Cl, an active-site-directed chloromethyl ketone that was designed for these studies, also produced strong inhibition. No evidence for either an essential sulfhydryl or a metal requirement was detected. The comparable behavior of the pulp and pituitary enzymes in all the inhibitor studies provided additional evidence for their common identity.

Lys-Pro-2-NNap, one of the new fluorigenic substrates resulting from this study, was hydrolyzed by (pituitary and pulpal) dipeptidyl peptidase II at a rate equal to that observed on the conventional assay substrate, Lys-Ala-2-NNap. Preliminary studies indicated that the new substrate has a lower K_m value. This characteristic, together with its anticipated resistance to degradation by (aminoacyl) aminopeptidases should make Lys-Pro-2-NNap a more sensitive and specific substrate for the assay of dipeptidyl peptidase II in impure preparations and, as the 2-(4-methoxy)naphthylamide derivative, for the localization of dipeptidyl peptidase II by electron microscopy. Lys-Ala-NNapOMe has already been used to demonstrate a lysosomal distribution for dipeptidyl peptidase II in the bovine anterior pituitary gland [1], the rat thyroid gland [3], the rat peritoneal macrophage [18] and according to our unpublished electron microscopic studies, in the fibroblasts of bovine dental pulp as well. The new Lys-Pro-NNapOMe substrate was recently made available to Drs. P. Sannes and B. Schofield at John Hopkins Medical Institutions. They have indicated (by personal communication) that a variety of collagen-synthesizing, connective tissue cells (i.e., fibroblasts, chondrocytes, osteocytes and osteoblasts) in the rat, exhibited intense staining for dipeptidyl peptidase II. Their electron microscopic studies revealed that the enzyme was confined to lysosomal structures in all cases. It thus appears that dipeptidyl peptidase II not only has a wide distribution, but also serves as a rather rare example of a lysosomal protease that belongs to the serine class.

Several investigators have described the presence of fragmented and partially-degraded collagen fibrils within the phagolysosomes of macrophages [19,20], fibroblasts located in oral connective tissues [21,22] and most recently fibroblasts of human pulp [23]. Ten Cate and coworkers [21,24,25] reported that intracellular collagen fibrils were commonly seen in the fibroblasts of oral connective tissues. These observations have been confirmed by Listgarten [22]

and Garant [26] and, together with other more recent studies [27] concerned with the role of fibroblasts in collagen turnover and resorption in the periodontal ligament, have led to the working hypothesis that intracellular collagen fibrils represent phagocytosed collagen destined to undergo enzymatic digestion within secondary lysosomes. The abundance of intracytoplasmic collagen fibrils within vacuoles of fibroblasts, especially those of the gingival and periodontal connective tissues [21,22], is consistant with studies [28,29] showing a high rate of collagen turnover in these tissues, probably the highest of any connective tissue in the body [5]. On the other hand, based on existing knowledge of the substrate specificities of the various lysosomal peptide hydrolases, it is not possible to attribute the cleavage of internal prolyl or hydroxyprolyl bonds (which comprise 20—25% of the bonds in collagen) to any known lysosomal enzyme, including those that have been shown to degrade native collagen in vitro at acidic pH, i.e., cathepsin B [30,31] and cathepsin N [32,33]. Based on the evidence described in the present communication, dipeptidyl peptidase II represents the first example of a lysosomal protease capable of cleaving peptide bonds on either side of proline.

The comparison of the activities of several lysosomal peptide hydrolases present in bovine dental pulp revealed that the fibroblast population of this connective tissue is especially rich in dipeptidyl peptidase II as judged by the level of its activity in relation to the DNA content of the tissue. Similar ratios of enzyme activity to DNA content were established for three other lysosomal proteases (dipeptidyl peptidase I, cathepsin B, and cathepsin D) in bovine pulp and in two other lysosome-rich tissues (bovine spleen and rat liver), commonly used as sources for the purification of lysosomal proteases. Relative to that of dipeptidyl peptidase II, the activities of dipeptidyl peptidase I and cathepsin B were surprisingly low in the fibroblasts of this connective tissue. On the other hand, a substantial quantity of a cathepsin D-like enzyme was found to be present in the fresh pulp extracts. This enzyme, which was originally detected in bovine pulp by Schwabe and Kalnitsky [34], has been purified [35] and its substrate specificity characterized [36]. When the activity of dipeptidyl peptidase II per mg tissue DNA was compared for bovine pulp, bovine spleen and rat liver, it was estimated that the pulpal fibroblasts were 6-fold and 3.2-fold richer in dipeptidyl peptidase II than the average cell comprising bovine spleen and rat liver. Even when compared on the basis of equal weight of fresh tissue, the content of dipeptidyl peptidase II in bovine pulp was 60% of bovine spleen and 91% of rat liver. Consequently, bovine pulp appears to be a relatively rich source of a typical dipeptidyl peptidase II whose purification will yield a lysosomal enzyme of fibroblast origin for further study of its possible role in collagen degradation.

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