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Studies on a Soluble Dipeptidase from Pig Intestinal Mucosa. Structural Properties

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A soluble dipeptidase (glycyl-L-leucine dipeptidase, EC 3.4.13.2) purified from pig intestinal mucosa was investigated for some of its structural properties. A molecular weight of 104 000 was determined under native conditions by gelfiltration but under denaturing conditions the molecular weight was 52 000 as determined by gelfiltration and polyacrylamide gel electrophoresis. These findings might suggest the native dipeptidase to be composed of two polypeptide chains of uniform molecular weight. The two polypeptide chains may be identical as only one N-terminal amino acid, proline, was found. The amino acid composition and the molar extinction coefficient (14.6×10^4 M^{-1} cm^{-1}) were determined. The soluble dipeptidase contained carbohydrate (about 0.3 %). The number of free SH-groups of the soluble dipeptidase was found to be about 5 when determined with *p*-hydroxymercuribenzoate but was higher (8 – 17) when determined with Ellman's reagent.

Little is known about the characteristics of the exopeptidases of the small intestine, a fact which is in contrast to the information available for the proteolytic enzymes of pancreas and ventricle. The purification and the specificity of a soluble dipeptidase (glycyl-L-leucine dipeptidase, EC 3.4.13.2) from the pig intestinal mucosa have earlier been reported.[1] The kinetics of the enzyme and the influence of some common enzyme inhibitors on its activity have been described.[2] In this paper the molecular weight and the amino acid composition are reported together with some other data on its structural properties.

MATERIALS AND METHODS

Enzyme. The soluble dipeptidase was prepared and tested for homogeneity as described earlier.[1] The enzyme preparation used had a specific activity of 1400 units [2] of activity per mg protein. It was stored (– 20°C) in 0.07 M sodium phosphate buffer containing 0.2 M NaCl, 4 mM 2-mercaptoethanol, and 12.5 % (w/v) glycerol and had a concentration of 0.4 mg per ml.

Chemicals. Bovine serum albumin was bought from Armour Pharmaceuphtical Co., Ltd., Eastbourne, England. Dansyl chloride, dansyl amino acids, acrylamide and *N,N'*-methylenebisacrylamide were purchased from BDH Chemicals Ltd., Poole, England. ε-Dansyl-lysine was a gift from Dr. Johan Stenflo, Malmö General Hospital, Malmö, Sweden. Yeast alcohol dehydrogenase was obtained from Calbiochem, San Diego, U.S.A. and bovine chymotrypsinogen from Mann Res. Labs., New York, U.S.A. HCl (suprapure), HBr (suprapure), 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) and Schiff's reagent were manufactured by Merck, Darmstadt, Germany. Blue Dextran 2000, Sephadex G-200, Sephadex G-25 Fine, and Sepharose 6B were bought from Pharmacia, Uppsala, Sweden, and sodium dodecyl sulfate (sequanal grade) from Pierce Chemical Co., Illinois, U.S.A. *E. coli* alkaline phosphatase (type III), sperm whale myoglobin, egg albumin, sodium *p*-hydroxymercuribenzoate (PHMB), milk xanthine oxidase (grade II), human transferrin, *nor*-leucine, cysteic acid, and methionine sulfone were bought from Sigma Chem. Co., St. Louis, U.S.A. Nitrogen (oxygen free) was a product of Norsk Hydro A/S Oslo, Norway. Other chemicals used were of analytical grade and the water used was distilled and de-ionized.

Determination of molecular weight

Native conditions. For the estimation of the molecular weight of the soluble dipeptidase under native conditions the method of Andrews [3] was used. A Sephadex G-200 column was loaded with a sample containing xanthine oxidase, alcohol dehydrogenase, alkaline phos-

phatase, egg albumin, and the soluble dipeptidase. The sample applied was 12.5 % (w/v) in respect to glycerol and was then applied to the column underneath the eluent. Fractions of about 2.5 ml were collected and their precise volumes determined by weight. The concentrations of alcohol dehydrogenase and egg albumin in the fractions were analyzed by measuring the absorbance at 230 nm (PMQ II Spectrophotometer, Zeiss, Oberkochen, Germany). The concentration of dipeptidase (substrate: glycyl-L-leucine), alkaline phosphatase (substrate: *p*-nitrophenyl phosphate) and xanthine oxidase (substrate: hypoxanthine) in the fractions were measured using established methods.[4-6]

Denaturing conditions. The molecular weight of the soluble dipeptidase was also estimated under denaturing conditions using polyacrylamide gel electrophoresis and gelfiltration in dodecyl sulfate. Before the experiments the soluble dipeptidase and the molecular weight standards (serum albumin, egg albumin, chymotrypsinogen, myoglobin) were treated in 1 % (w/v) dodecyl sulfate and 1 % (v/v) 2-mercaptoethanol for 2 min at 100 °C.[7] In some experiments the soluble dipeptidase was pretreated with dodecyl sulfate only under the same conditions.

The polyacrylamide gel electrophoresis in dodecyl sulfate was performed essentially as described by Weber *et al.*[7] using a total monomer concentration in the gel of 10 % (w/v). The *N,N'*-methylenebisacrylamide was 1 % (w/v) of the total monomer concentration. About 20 μg of each protein were applied and the gels were stained with Coomassie Brilliant Blue. Three parallel gels were run in each experiment, one containing the molecular weight standards, a second the soluble dipeptidase, and a third the molecular weight standards and the soluble dipeptidase together.

Gelfiltration in dodecyl sulfate [8] was performed using Sepharose 6B. The column was eluted using a peristaltic pump (Varioperpex, LKB Produkter AB, Bromma, Sweden). Fractions of equal volume were collected each 15 min and their absorbance measured at 230 nm. The void volume in every experiment was determined by Blue Dextran. Before and after the experiments with the enzyme the column was calibrated with the molecular weight standards (100 μg of each applied).

Determination of amino acid composition and E(1 %, 1 cm) value

A solution of the dipeptidase (1 ml) was thoroughly dialyzed, (dialysis tubes, Visking Co., Chicago, U.S.A.) against 3 × 500 ml 0.05 M *N*-ethylmorpholine-acetate buffer (pH 8.5). The absorbance at 280 nm of the dialyzed sample was measured with the used dialysis buffer as a reference and the sample was then transferred to three different ampoules (300 μl in each). After lyophilization of the samples, 1 ml 6 M HCl and a crystal of phenol were added.[9] The ampoules were flushed with nitrogen and after a thorough evacuation [10] they were closed and hydrolyzed for different times (24, 48, 72 h) at 110 °C. After performed hydrolysis the HCl was removed, using a rotoevaporator (40 °C). The amino acid analysis was performed on an amino acid analyzer (Multichrom, Model 4255, Beckman, Munich, Germany) using the dual column system.[10]

The E(1 %, 1 cm) value was calculated from the absorbance and the amino acid analysis data using the finally calculated amino acid composition.

The amount of the sulfur containing amino acids of the soluble dipeptidase was determined using the oxidation with performic acid.[11] A solution of dipeptidase solution (300 μl) was dialyzed as described above. The sample was lyophilized, dissolved in 100 μl formic acid and 2 ml performic acid was added. As no precipitates were observed during the oxidation the process was interrupted after 4 h by 300 μl HBr (47 %) and then 15 ml of water was added and the sample was lyophilized. The hydrolysis (24 h) was performed as described above. The amount of cysteic acid and methionine sulfone formed was determined using the amino acid analyzer and their content was related to the leucine content. The yield of cystine (20 nmol) in the procedure was determined in separate experiments.

The tryptophan content was determined spectrophotometrically using the method of Edelhoch.[12] A dipeptidase solution (300 μl) was first dialyzed against 2 l 0.02 M sodium phosphate buffer (pH 6.5) and then against 200 ml 6 M guanidine chloride (Norit treated) in the same buffer. The absorbance of the sample was measured using the last dialysis buffer as a reference and afterwards given norleucine (50 nmol). It was then hydrolyzed (24 h) and analyzed for its amino acid content. The protein content was calculated both from the absorbance data and from the amino acid analysis.

Determination of N-terminal amino acid

The end-group analysis of the soluble dipeptidase was performed using the dansyl chloride method.[13] Prior to the reaction with dansyl chloride, the dipeptidase solution (300 μl) was made 1 % (w/v) in respect to dodecyl sulfate and 2-mercaptoethanol according to Weiner *et al.*[14] The dipeptidase solution was then dialyzed overnight against a 0.2 M *N*-ethylmorpholine-acetate buffer (pH 8.5) containing 0.16 % dodecyl sulfate and lyophilized. The lyophilized sample was dissolved in water and *N*-ethylmorpholine (50 μl of each) and then 75 μl of a dansyl chloride solution (25 mg/ml dimethylformamide) was added. The reaction

was interrupted after 3 h by 500 μl ice-cold trichloroacetic acid (20 % w/v) added drop by drop. The formed precipitate was spun down and washed four times with 500 μl 1 M HCl each time to get rid of the dansylic acid. To the dried precipitate 50 μl 6 M HCl was added and the sample was hydrolyzed for 16 h. After performed hydrolysis the HCl was removed in a rotoevaporator. The sample was then dissolved in 10 μl acetone-acetic acid (3:2, by vol.) and subjected to chromatography on polyamide sheets (Cheng Chin Trading Co., Taipei, Taiwan). The chromatographic systems used were water-90 % formic acid (200:3, by vol.) and benzene-acetic acid (9:1, by vol.).[15] In order to separate dansyl-arginine, dansyl-histidine, and ε-dansyl-lysine the additional solvent systems ethyl acetate-methanol-acetic acid (20:1:1, by vol.) and 0.05 M trisodium phosphate-ethanol (3:1, by vol.)[16] were run.

Determination of number of free SH-groups

The number of free SH-groups of the soluble dipeptidase was determined using Ellman's reagent [17] and PHMB.[18]

Prior to the reaction with Ellman's reagent 2-mercaptoethanol was removed from the dipeptidase solution using either dialysis against 3 × 1 l 0.05 M Tris-HCl buffer (pH 8.1), containing 10 mM EDTA or gelfiltration on a Sephadex G-25 column (0.9 cm × 11.7 cm) equilibrated in the same buffer. The absorbance of the sample at 280 nm was measured (0.15–0.30). To 300 μl of the sample were then added 10 % (w/v) dodecyl sulphate solution (15 μl) and Ellman's reagent (25 μl, 5 mg/ml methanol). The course of the reaction was followed by measuring the absorbance at 412 nm during a 30 min period and the concentration of free SH-groups was calculated using a molar extinction coefficient of 13 600 M^{-1} cm^{-1} for the 3-carboxylato-4-nitro-thiophenolate ion.[17]

Before the reaction with PHMB the dipeptidase solution (150 μl) was dialyzed overnight against 0.1 M sodium phosphate buffer (pH 7.5). The solution was measured for its absorbance at 280 nm, made 0.5 % (w/v) in respect to dodecyl sulfate and then added 75 nmol PHMB. The reaction was allowed to proceed overnight at room temperature. The mercury content of the dipeptidase was measured by a method developed in our laboratory using an atomic absorption spectrophotometer (Model 103, Perkin-Elmer, Norwalk, Connecticut, U.S.A.), equipped with a flameless mercury analysis system (Carlsen, J. B. *Anal. Biochem. In press*).

Determination of carbohydrate content

The carbohydrate content of the soluble dipeptidase was analyzed by polyacrylamide gel electrophoresis in combination with Schiff's reagent.[19] About 75 μg of the soluble dipeptidase was run on two parallel gels each. After completion of the electrophoresis, one gel was stained with Coomassie Brilliant Blue and the other one with Schiff's reagent. A dilution series of transferrin was run in parallel in order to get a semiquantitative estimation of the carbohydrate content of the dipeptidase.

RESULTS AND DISCUSSION

The molecular weight of the soluble dipeptidase was estimated to be 104 000 under native conditions (Fig. 1), assuming it to be globular and of the same grade of hydration as the molecular weight standards. In comparison to earlier reported molecular weight data on dipeptidases this value is close to that found for the purified intestinal monkey dipeptidase [20] but somewhat higher than observed for the mouse ascites tumor dipeptidase [21] and much higher than that reported for the renal dipeptidase.[22]

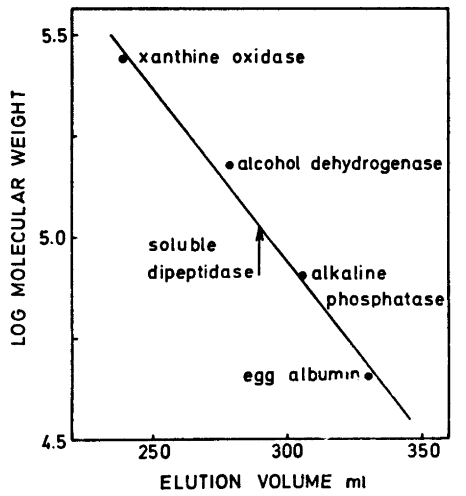

Fig. 1. Determination of the molecular weight of the soluble dipeptidase on Sephadex G-200. The column (2.5 cm × 93 cm) was equilibrated in and eluted with 0.1 M sodium phosphate buffer (pH 7.0), containing 4 mM 2-mercaptoethanol, at a constant flow rate of 5 ml/h. The sample (2.7 ml) applied contained 150 μg soluble dipeptidase.

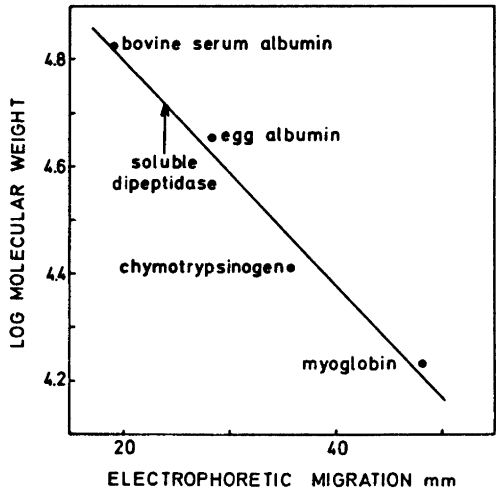

Fig. 2. Determination of the molecular weight of the soluble dipeptidase under denaturing conditions using polyacrylamide gel electrophoresis. The experiment was performed applying 8 mA per gel for 4 h.

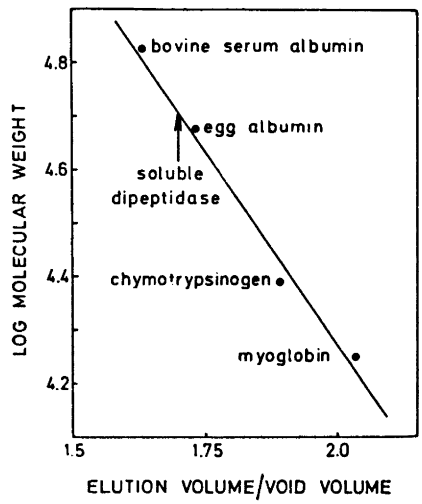

Fig. 3. Determination of the molecular weight of the soluble dipeptidase under denaturing conditions using gelfiltration on Sepharose 6B (room temperature). The column (0.9 cm × 57 cm) was equilibrated in and eluted with 0.07 M sodium phosphate buffer (pH 7.2), containing 1 % dodecyl sulfate, at a constant flow rate of 1.75 ml/h.

The molecular weight estimated for the reduced and dodecyl sulfate treated enzyme when determined by gel electrophoresis was 52 000 (Fig. 2). Irrespective of the enzyme being reduced and dodecyl sulfate treated or only dodecyl sulfate treated, the gel electrophoresis resulted in only one dominating band with a constant position, when the soluble dipeptidase was run alone. Likewise one peak corresponding to a molecular weight of 51 000 (Fig. 3) was obtained in the experiments on Sepharose 6B, irrespective of which of the two pre-treatments that had been used.

These molecular weight data may suggest the soluble dipeptidase to be composed of two polypeptide chains with a uniform molecular weight and associated with non-covalent bonds. Since N-terminal amino acid analysis revealed proline only, the two polypeptide chains might be identical.

The amino acid composition of the soluble dipeptidase is given in Table 1. Figures are given for mol % and for the number of amino acids per molecule of enzyme using a molecular weight of 104 000. The yield of cystine in the oxidation experiments with performic acid was determined to 83 % and this figure was used in the calculations. The cysteic acid and methionine sulfone content obtained in the amino acid analysis was related to the other amino acids by means of the leucine content. The figure for the tryptophan content was obtained by relating it to the leucine content and to the absorbance at 280 nm. In both cases a value of 18.4 mol of tryptophan per mol of dipeptidase was obtained.

A value of 14.0 for E(1 %, 1 cm) was found. Using a molecular weight of 104 000 the molar extinction coefficient was calculated to be 14.6×10^4 M^{-1} cm^{-1}.

The Ellman reaction with the soluble dipeptidase was rapid and complete within 10 min but the results obtained varied. When the 2-mercaptoethanol was removed by gelfiltration the reaction gave 12 – 17 mol SH-groups per mol dipeptidase while removal of the 2-mercaptoethanol by dialysis resulted in 8 – 11 mol SH-groups per mol dipeptidase. When the dipeptidase was analyzed with the PHMB-reagent only 4.8 – 5.1 mol SH-groups per mol enzyme were found showing that this reagent is less reactive to the soluble dipeptidase than Ellman's reagent. The number of free SH-groups obtained indicates that the soluble dipeptidase contains only few disulfide bonds, if any.

Table 1. Amino acid composition of the soluble dipeptidase. The values of the stable and quantitatively liberated amino acids are given as the mean of the results from the three different hydrolysis times with the coefficient of variation. The number of residues per molecule dipeptidase is calculated on the basis of a molecular weight of 104 000.

Amino acid	Mol %	Coefficient of variation (%)	Number of residues per molecule dipeptidase
Lys	7.05	3.9	66
His	2.56	5.3	24
Arg	3.76	3.5	35
Asx	10.11	0.77	94
Thr [a]	4.32	–	40
Ser [a]	6.06	–	57
Glx	11.03	1.3	103
Pro	4.84	2.2	45
Gly	8.87	4.7	83
Ala	6.89	0.78	64
Cys [b]	1.79	–	17
Val [c]	6.87	–	64
Met [b]	1.95	–	18
Ile [c]	5.47	–	51
Leu	9.40	1.4	88
Tyr [a]	3.60	–	34
Phe	3.55	5.6	33
Trp [d]	1.97	–	18
Total number of residues			934

[a] Extrapolated to zero time by assuming destruction according to a first order reaction.[10] [b] Values obtained after performic acid oxidation.[11] [c] Values obtained from 72 h hydrolysis. [d] Determined spectrophotometrically.[12]

By comparing the intensity of the color obtained with the Schiff reagent on the band of the soluble dipeptidase with the intensity of the bands obtained for transferrin, the carbohydrate content of the enzyme was estimated to be 0.3 %. Recently, the carbohydrate content of another pig intestinal peptidase has also been reported.[23]

A comparison with the prolidase recently reported purified from pig intestinal mucosa [24] and now investigated for its structural properties [25] shows the same molecular weight and subunit structure. It has a similar carbohydrate content. There are, however, some differences in the amino acid composition of the two enzymes. The content of tyrosine and tryptophan of the soluble dipeptidase is higher than that found for the prolidase. This finding is consistent with the differences found in the $E(1\ \%, 1\ \text{cm})$ values for the two enzymes. The two enzymes also display differences in their covalent structure, which may explain the differences in stability of the enzymes and in their reactivity to Ellman's reagent.

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