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ON PORCINE ENTEROKINASE. FURTHER PURIFICATION AND SOME MOLECULAR PROPERTIES*

J. BARATTI, S. MAROUX, D. LOUVARD AND P. DESNUELLE
Centre de Biochimie et de Biologie Moléculaire du C.N.R.S. 31, Chemin J. Aiguier 13009 Marseille (France)

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SUMMARY

Enterokinase has been purified more than 1000-fold from porcine duodenal mucosa. The final preparations appear to be homogeneous by disc electrophoresis and immunodiffusion assays, and to be entirely free of aminopeptidase activity. Starting with 20 kg of duodenum (5 kg of mucosa), about 75 mg (0.4 μmole) of the pure enzyme can be obtained, with a 26% yield.

Since enterokinase is a glycoprotein with an unusually high sugar content (neutral sugars, 20%; amino sugars, 15%; sialic acids, 2%; total, 37%), several techniques were applied for the determination of its molecular weight. Although all techniques gave similar values, the most likely was considered to be 195 000. The only visible effect of the bulky sugar part was to significantly lower the partial specific volume of the protein (0.705 g/ml).

The enterokinase molecule (about 1100 amino acid residues) is not composed, like other proteins of similar molecular weight, of several subunits linked by non-covalent bonds. However, upon reduction it gives rise to 2 chains, the "heavy" and the "light" chain, with molecular weights of 134 000 and 62 000, respectively (about 700 and 350–400 amino acid residues). The sugar content of both chains is approximately the same.

One active serine per mole can be titrated by [^{32}P]DFP in pure enterokinase preparations. This serine is located in the light chain.

INTRODUCTION

A number of years ago, it was reported by Kunitz[1,2] that partially purified preparations of an intestinal enzyme called enterokinase (enteropeptidase, EC 3.4.4.8) could readily activate trypsinogen, at pH 5.6, by a first-order reaction. Yamashina[3–5]

Abbreviations: S-CM, *S*-carboxymethyl; DFP, diisopropylfluorophosphate; DP-enzyme, diisopropylphosphorylenzyme derivative; BAEE, *N*-benzoyl-L-arginine ethylester.

* This work is a part of the Dr. Sc. thesis of J. Baratti, Faculty of Sciences, Marseilles, 1973.

showed later that enterokinase is a glycoprotein and that it activates trypsinogen through the cleavage of the same lysine–isoleucine bond as trypsin during autoactivation.

More recently, highly purified enterokinase preparations of porcine origin were obtained in this laboratory by a combination of ion-exchange chromatography and gel filtration[6]. The specificity of the enzyme was investigated for the first time and found to be very narrowly restricted to the characteristic polyaspartyl-lysine structure present in the N-terminal region of trypsinogen immediately before the "strategic" bond Lys–Ile. This finding taken jointly with the known fact that pancreatic zymogens are entirely unactivated in the duodenum of children with a congenital enterokinase deficiency[7], led to the conclusion that enterokinase is a key enzyme for the intraluminar digestion of proteins *in vivo*.

A new technique for the purification of this enzyme from porcine duodenal mucosa has now been worked out. Its main advantage is to ensure a complete removal of aminopeptidase and to provide the comparatively high amounts of pure enzyme necessary for structural investigations (molecular weight, absence of non-covalently linked subunits, presence of 2 chains of unequal length bound by disulfide bridges, amino acid and sugar composition).

METHODS

Enzymatic activities

The technique previously employed[6] for the determination of enterokinase through the activating effect of this enzyme on trypsinogen (Worthington, once crystallized, potential specific activity against *N*-benzoyl-L-arginine ethylester (BAEE), 45 μmoles$\cdot$min$^{-1}\cdot$mg^{-1}), was modified in order to take into account the strong influence of ionic strength on the reaction rate. Details and full justification of the new technique will be published in the near future. For the assays, the following reagents were mixed: 1 ml of a 70-mM succinic acid–sodium succinate buffer, at pH 5.6, (instead of the 50 mM citric acid–sodium citrate buffer at the same pH used previously); 0.5 ml of a 1 mg/ml aqueous solution (5 mM in $CaCl_2$) of bovine trypsinogen (Worthington, once crystallized), x ml of the enterokinase-containing solution and $1-x$ ml of water. After a 30-min incubation period at 25 °C, the reaction was stopped by the addition of 50 μl of 2 M HCl. Trypsin activity was evaluated potentiometrically on 0.5–1.0-ml aliquots with the aid of a recording Radiometer pH-stat and BAEE as the substrate. The specific activity of the pure bovine enzyme against BAEE was found to be 50 μmoles$\cdot$min$^{-1}\cdot$mg^{-1}. One enterokinase unit (trypsinogen) is defined as the amount of enzyme activating 1 nmole of the zymogen within 30 min under the conditions of the assays. Enterokinase activity was also directly tested on BAEE. The conditions of the assays were: substrate concentration, 20 mM; pH 7.9; 25 °C. One enterokinase unit (BAEE) is the amount of enzyme hydrolyzing 1 μmole of BAEE per min under these conditions.

Aminopeptidase activity was measured with the substrate L-alanine-*p*-nitroanilide according to a recently described method[8]. An aminopeptidase unit was defined as the amount of enzyme generating 1 nmole of *p*-nitroaniline from this substrate per min under the conditions of the assays.

Determination of the concentration of enterokinase solutions

This was carried out spectrophotometrically at 280 nm, using a value of 8.6 (average of 2 independent assays which gave 8.5 and 8.7, respectively) for the extinction coefficient ($E_{1\,cm}^{1\%}$) of the pure enzyme at this wavelength. For the determination of the $E_{1\,cm}^{1\%}$, enzyme solutions were split into 2 parts: the first served for the spectrophotometric measurement and the second was vacuum-dried over P_2O_5 until constant weight (about 24 h).

Sugar composition

Neutral sugars, amino sugars and sialic acid were evaluated by the phenol–sulfuric acid method, the Elson–Morgan technique and the barbituric acid reaction, respectively[9]. Glucosamine and galactosamine could also be measured in the amino acid analyzer after a special hydrolysis of the protein in 5.7 M HCl at 115 °C for 6, 15 and 24 h. Results were extrapolated to zero time.

Reduction–carboxymethylation

Enterokinase was reduced by a 4-h incubation period at room temperature with 0.1 M β-mercaptoethanol in a 0.36 M Tris–HCl buffer (pH 8.6), 8 M in urea and 0.2% in EDTA (ref. 10). The reduced protein was carboxymethylated by treatment with iodoacetic acid according to the usual technique.

Immunochemical determination of aminopeptidase

Antiserum was raised in rabbits by intradermal injection of antigen in complete Freund's adjuvant. The amount of injected material was 20 mg of the aminopeptidase-containing brown powder resulting from the first DEAE-cellulose chromatography (see Results), and 6 mg of an intestinal aminopeptidase preparation recently purified in this laboratory (unpublished).

Amino acid analysis

This analysis was carried out by the technique of Spackman[11] with the aid of a Beckman amino acid analyzer Model 120-C. Enterokinase samples or the S-carboxymethylated derivatives of the H and L chains (see later) were dissolved (1 mg/ml) in 5.7 M triple-distilled HCl and hydrolyzed in duplicate for 24, 48 and 72 h at 115 °C. Norleucine was added to the hydrolyzate as an internal standard. Serine and threonine were calculated by extrapolation to zero time. Tryptophan was determined colorimetrically on intact enterokinase. Half-cystine was evaluated, either as cysteic acid after performic acid oxidation of enterokinase or as *S*-carboxymethyl (S-CM)-cysteine in the H and L chains (see text). Methionine was also evaluated as methionine sulfone in performic acid-oxidized enterokinase.

As already mentioned earlier, glucosamine and galactosamine could be measured in the autoanalyzer and were well separated from each other under the normal conditions of column elution, emerging just after phenylalanine.

Electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate

Electrophoresis assays were carried out in 5% polyacrylamide gels according to the method of Weber and Osborn[12]. Enterokinase samples and the reference proteins were dissolved in a 0.1 M phosphate buffer (pH 7.2) containing 1% sodium

dodecyl sulfate, and the solutions were incubated for 18 h at 25 °C or for 15 min at 60 °C. When required, β-mercaptoethanol was also added to the buffer at a 1% concentration in order to reduce the disulfide bridges of the proteins. However, better results were obtained in this respect when the S-carboxymethylated derivatives of the proteins were employed. The relative migration distances of the bands (electrophoretic mobility) were corrected for gel shrinkage during staining and destaining, and then plotted against the logarithm of the corresponding molecular weights. The following reduced or unreduced reference proteins were used for the establishment of the calibration curves: bovine trypsinogen (Worthington, once crystallized; molecular weight: 24 000); bovine chymotrypsin (Worthington 3 times crystallized; 24 500); porcine pepsin (crystallized in the laboratory; 34 000); ovalbumin (sample crystallized in the laboratory; 43 000); catalase (Worthington 2 times crystallized; 60 000); bovine serum albumin (Mann Research crystallized, 100% by electrophoresis; 69 000); β-galactosidase from *Escherichia coli* (Worthington, chromatographically purified; 130 000).

Gel filtration in dissociating and non-dissociating media

Filtrations in the absence of dissociating agents were carried out in 2 cm × 200 cm Sephadex G-200 columns equilibrated and eluted with a 10 mM Tris–acetate buffer (pH 6.0), 0.5 M in NaCl. Blue dextran and *N*-dinitrophenylalanine were used for the evaluation of the void and total volume of the column, respectively. The column was calibrated using the same proteins as used for gel electrophoresis, *plus* human γ-globulin (molecular weight, 150 000), kindly provided by Professor M. Fougereau.

Two techniques were used for operating in a dissociating medium. In the first, a 1.5 cm × 90 cm Sepharose 4B column was equilibrated with 0.1 M phosphate buffer (pH 7.2) containing 1% dodecyl sulfate. The proteins or their S-carboxymethylated derivatives (0.5 mg) were incubated at 60 °C for 2 h with 1 ml of the buffer and the solutions were pumped in at the bottom of a column equipped with adaptors for ascending chromatography. The protein content of the eluant was continuously recorded at 230 and 280 nm with an Elugraph monitor (Seive, Paris). The second technique was similar to that described by Tanford[13]. Sepharose 4B was equilibrated with a 6 M guanidine·HCl–HCl buffer at pH 7.2. The slurry was carefully degassed for 4 h under vacuum and poured into a 1-cm wide column to form a 90-cm-high gel bed. The proteins or their S-carboxymethylated derivatives (2–3 mg) were dissolved in 0.5 ml of the buffer. After a 1-h incubation at 25 °C, the solutions were pumped in as above at the bottom of the column. The same proteins as above were employed for column calibration.

RESULTS

Improved method for the purification of porcine enterokinase

Porcine enterokinase is now known to be bound to the brush-border membrane of the duodenum[14]. Therefore, the starting material for its purification should not be the duodenal content as claimed earlier[1], but the mucosa. Porcine duodenum (20 kg) was collected in the slaughterhouse immediately after the death of the animals, then frozen at −20 °C and transported to the laboratory where it was

allowed to thaw. The mucosa was immediately extruded by passage through two closely adjusted rubber rollers and the resulting slurry (5 kg) was stored in the frozen state until use. All other steps were carried out at 4 °C unless otherwise stated.

Membrane-bound enterokinase has recently been reported to be solubilized by bile salts[15]. To explore this possibility, a sodium deoxycholate solution was prepared by dissolving deoxycholic acid (purum; Fluka, Switzerland or technical grade; Expandia, France) at a concentration of 25% (w/v), in 1 M NaOH accompanied by stirring. This solution was mixed with a suspension of mucosa in water. The volumes were adjusted so as to twice dilute the mucosa and give final deoxycholate concentrations of 0.5, 1.0, 1.5, 2.0 or 2.5%. The final pH was set at 8.0. After a 4-h incubation, the mixtures were centrifuged at 105 000 × *g* for 3 h in a Beckman L2-50 centrifuge in order to separate the soluble form of the enzyme (supernatant) from the bound form (sediment). Enterokinase was found to be largely solubilized by 1% deoxycholate, but, in order to avoid difficulties during the subsequent steps, a 2.5% concentration was used throughout. Approximately 84% of the total enterokinase was found in the 105 000 × *g* supernatant under these conditions, as compared to 33% when deoxycholate was omitted. A substantial part of the activity remaining in the 105 000 × *g* pellet in the presence of deoxycholate may be recovered by washing.

On a preparative scale, 2.5-kg amounts of the thawed mucosa were incubated for 1 h with 2.5% deoxycholate at pH 8.0 and 0 °C. After dialysis to remove deoxycholate, the pH was adjusted to 6.0 by glacial acetic acid accompanied by stirring. Acidification to pH 5.0, as recommended in our first method[6], was avoided because of the noticeable inactivation undergone by enterokinase at this pH. The insoluble material was removed by a 30-min centrifugation at 27 000 × *g* in a Sorvall refrigerated centrifuge Model RC2B and the clear supernatant (pH 6.0 supernatant; about 8 l) was pumped into a 9 cm × 30 cm column of DEAE-cellulose (Whatman DE 11) equilibrated with a 10 mM Tris–acetate buffer (pH 6.0), containing 50 mM NaCl. The unretarded proteins and those washed away by passage of 5 l of the equilibration buffer, were discarded and the remainder (about 30% of the total), containing enterokinase, was eluted at the same pH by a linear 50–400 mM NaCl concentration gradient.

Fig. 1 shows that enterokinase emerges from the DEAE-cellulose column just ahead of a large protein peak, at a NaCl concentration of approximately 0.15 M. Fractions 90–150, indicated by an horizontal bar, were pooled, dialyzed and lyophilized. The resulting brown powder was dissolved in 100 ml of a 10 mM Tris–acetate buffer (pH 6.0), containing 0.5 M NaCl and the solution was passed through a 6 cm × 200 cm Sephadex G-100 column, equilibrated and eluted by the same buffer. Enterokinase was not retarded in this system. The corresponding fractions were dialyzed and lyophilized. Enterokinase was separated from trypsin at this point as indicated by a sharp increase (from 0.22 to 3.3) of the activity ratio of the solutions towards BAEE and *N*-tosyl-L-arginine methylester. This ratio is known to be 3.5 for pure enterokinase[6], whereas it does not exceed 0.1 for trypsin.

Next, two preparations were pooled, dissolved in water, cooled at 0 °C and acidified to pH 4.0 by careful addition of glacial acetic acid accompanied by stirring. After 1 h of standing, the mixture was centrifuged at 27 000 × *g* for 30 min to remove a brown precipitate and the pH of the colorless supernatant (pH 4.0 supernatant)

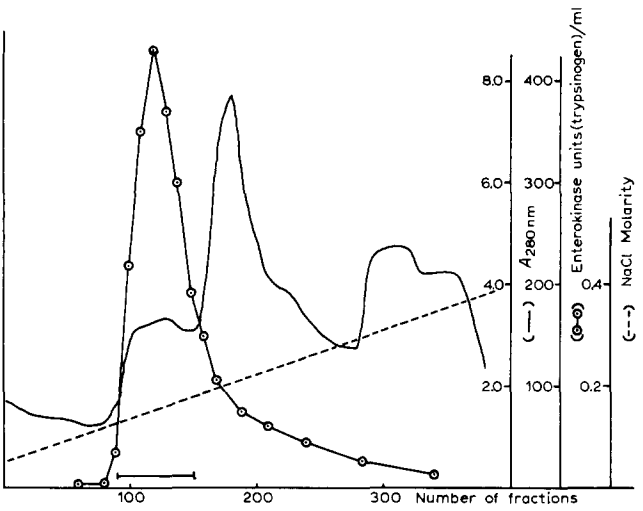

Fig. 1. First DEAE-cellulose chromatography. The volume of the liquid in the two chambers serving for the 50–400-mM gradient formation was 5 l. Elution rate, 200 ml/h. Volume of fractions, 30 ml. In these assays, enterokinase was evaluated through its activating effect on trypsinogen.

was quickly raised to 6.0 by the addition of 1 M NaOH. The resulting solution, made 10 mM in Tris–acetate buffer (pH 6.0) and 50 mM in NaCl, was pumped into a 9 cm × 10 cm DEAE-cellulose column equilibrated with the same buffer. The column was washed with 500 ml of the buffer and it was eluted by a linear NaCl concentration gradient from 50 to 400 mM. The elution profile was not essentially different from that already reproduced in Fig. 2 of our previous report[6]. The main contaminant, aminopeptidase, emerged just before enterokinase and was partly separated. The fractions under the enterokinase peak were pooled, dialyzed and lyophilized. The powder was dissolved in 50 ml of the 10 mM Tris–acetate buffer (pH 6.0), 0.5 M in NaCl and filtered through a 4 cm × 600 cm Sephadex G-200 column equilibrated with the same buffer. Fig. 2 indicates that enterokinase moves slightly faster than aminopeptidase in this system.

The fractions indicated by a horizontal bar in Fig. 2 were dialyzed and lyophilized. The final preparations were found to be homogeneous by disc electrophoresis (Tris–glycine buffer (pH 8.5) and 7.5% polyacrylamide gel). The double diffusion technique of Ouchterlony[16] gave a single line of precipitation when antisera against the brown powder obtained after the first DEAE-cellulose column was used. This antisera could be expected to contain antibodies against several intestinal proteins and consequently to give several lines if the preparation was impure. Moreover, no precipitation was observed when the antiserum prepared with pure aminopeptidase was used. The preparations were also free of any detectable aminopeptidase activity.

A balance sheet of the procedure is presented in Table I. Starting with 20 kg of porcine duodenum (5 kg of mucosa), an average amount of 75 mg of pure enterokinase was obtained. The purification effect calculated from the pH 6.0 supernatant was 1000-fold on the average and the yield was 26%.

TABLE I

BALANCE SHEET OF THE PURIFICATION OF ENTEROKINASE FROM 20 kg OF PORCINE DUODENUM

Steps	*Number of enterokinase units* $\times 10^6$	*Yield (%)*	*Specific activity towards trypsinogen*	*Purification (times)*
pH 6.0 supernatant	12.9	100	42	1
First DEAE-cellulose chromatography	10.0	77	940	22
Sephadex G-100 filtration	7.5	58	1 800	42
pH 4.0 supernatant	6.3	49	2 300	53
Second DEAE-cellulose chromatography	4.8	37	10 000	242
Sephadex G-200 filtration	3.4	26	44 000*	1004

* Due to several modifications in the technique used for the determination of enterokinase activity (lower ionic strength, presence of Ca^{2+}; see text), this figure is approximately 8 times higher than that previously given for the pure enzyme[6]. The corresponding value of the specific activity towards BAEE is 8.0.

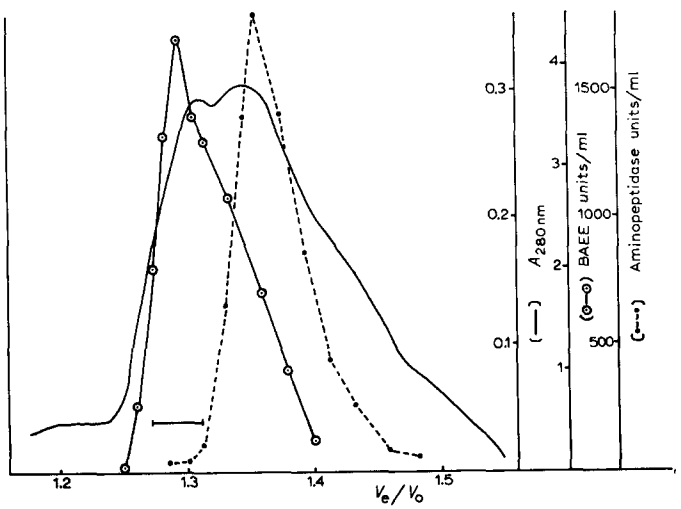

Fig. 2. Separation of enterokinase and aminopeptidase by a filtration through a 4 cm × 600 cm column of Sephadex G-200 in a 10 mM Tris-acetate buffer (pH 6.0), 0.5 M in NaCl. The void volume of the column was 4 l. Elution rate, 12 ml/h. Volume of fractions, 20 ml.

Preparation of diisopropyl-[^{32}P]-phosphoryl-enterokinase and active-site titration

Enterokinase has already been shown in this laboratory to be readily inhibited by diisopropylfluorophosphate (DFP) and *N*-α-tosyl-L-lysine chloroketone. Pure enzyme samples (specific activity with BAEE, 8.0) were incubated for 3 h with 10^{-4} M [^{32}P]DFP (Amersham, England, specific radioactivity, 50 Ci/mole), according to a previously described method[6]. After dialysis, the mixture was passed through a 2 cm × 200 cm Sephadex G-200 column equilibrated with 10 mM Tris–acetate buffer (pH 6.0), containing 0.5 M NaCl. The resulting pattern showed a single peak with a constant specific radioactivity. The number of phosphorus atoms per mole of enzyme was calculated by reference to α-chymotrypsin (Worthington, 3 times

crystallized) and found to be 0.97, assuming a molecular weight of 195 000 for the enzyme (see later). These data show that enterokinase contains a single active site per mole and that our purest preparations are free of any inert contaminant.

Use of a calibrated column of Sephadex G-200 in a 10 mM Tris–acetate buffer (pH 6.0) 0.5 M in NaCl, also permitted the evaluation of the molecular weight of native enterokinase and its diisopropyl-[^{32}P]-phosphoryl-([^{32}P)]DP)-derivative. In both cases, a value of 212 000 was found.

Ultracentrifugation assays

Assays were conducted either at high speed for evaluating the sedimentation coefficient of enterokinase, or at low speed for the determination of the molecular weight of the enzyme by equilibrium centrifrigation in a short column according to yphantis[17]. For the sedimentation coefficient, pure enterokinase was dissolved at 0.5% concentration in the 10 mM Tris–acetate buffer (pH 6.0), 0.5 M in NaCl. The solution was dialyzed for 48 h against the buffer and then centrifuged for 88 min at 60 120 rev./min and 20 °C in a Spinco-Beckman analytical ultracentrifuge Model E equipped with a scanner. As shown by Fig. 3, no sign of heterogeneity could be detected under these conditions. The sedimentation coefficient ($s_{20,w}$) was found to be 8.14 S.

In another series, enterokinase was sedimented in the strongly dissociating 6 M guanidine·HCl–HCl buffer at pH 7.2 and a value not exceeding 5.04 S was obtained under these conditions, appropriate corrections were made for the density and viscosity of the solution. Since the absence in enterokinase of subunits linked by non-covalent bonds has been subsequently demonstrated by a variety of other techniques (see later), no explanation for this puzzling effect can be given here.

Moreover, when 1% β-mercaptoethanol was added to the enterokinase solution in 6 M guanidine, two peaks separated, corresponding to $s_{20,w}$ values of 3.2 and 2.2 S, suggesting that two polypeptide chains bound to each other by disulfide bridges were present in enterokinase.

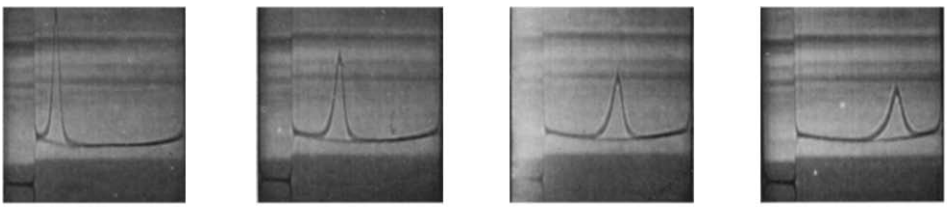

Fig. 3. Sedimentation pattern of enterokinase. Photographs were taken 16, 32, 48 and 64 min (left to right) after reaching maximal velocity of the rotor.

Considering the high sugar content of enterokinase (about 40%, see later), it was anticipated that calculation of the partial specific volume of the protein from its amino acid constituents alone would lead to serious errors. Accordingly, this parameter was measured with the aid of a specially designed microdensitometer (Model 120-C; A. Parr, Austria) and was found to be 0.705 g/ml in the 10 mM buffer used for centrifugation experiments. Two independant equilibrium ultracentrifugation assays were then undertaken with 0.8 mg/ml solutions in the same buffer. Strictly

linear plots were obtained in both cases from which molecular weight values of 193 000 and 195 700 could be derived. An average value of 194 000 for native enterokinase was obtained.

For some unknown reason, all our attempts to evaluate the molecular weight of enterokinase by ultracentrifugation in the presence of 6 M guanidine gave erratic results, thus these experiments have necessarily been discontinued.

Gel electrophoresis in 1% sodium dodecyl sulfate

In a first series of assays, the enzyme and the reference proteins were directly dissolved (1 mg/ml) in a 10 mM phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate. After standing at 25 °C for 18 h or at 60 °C for 15 min, the solutions were subjected to electrophoresis under standard conditions. Since unreduced proteins had not yet been used for the calibration of electrophoresis plates, it is of interest to observe (see Fig. 4) that a satisfactory linear reference plot of the logarithms of the molecular weight *vs* electrophoretic mobility could be obtained under these conditions. As already reported by others, bands corresponding to the dimer and trimer of bovine serum albumin were visible on the plates and greatly improved the accuracy of the method in the high molecular weight range. Catalase also gave rise to several bands, with a major one at the position expected for a molecular weight of 60 000.

The excellent agreement existing between the values given for intact enterokinase by gel electrophoresis in sodium dodecyl sulfate (195 000), gel filtration (212 000) and ultracentrifugation in a non-dissociating medium (194 000) definitely ruled out the possibility of enterokinase being composed of several subunits linked by non-covalent bonds.

In a second series, the gel was calibrated with the *S*-carboxymethyl (S-CM)-proteins. As seen in Fig. 4, results thus obtained did not significantly differ from those reported above. Enterokinase was, either incubated for 18 h at room temperature with 1% β-mercaptoethanol prior to electrophoresis, or S-carboxymethylated.

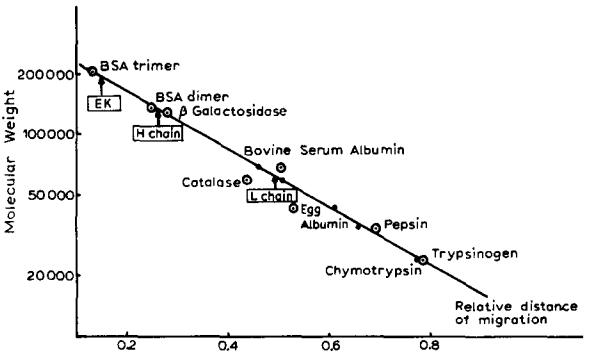

Fig. 4. Molecular weight of intact enterokinase (EK) and related chains (heavy (H) and light (L)) by electrophoresis in the presence of 1% sodium dodecyl sulfate. The logarithms of the molecular weights of the reference proteins are plotted *vs* the observed electrophoretic migration. Open circles, unreduced proteins; closed circles, S-CM-proteins. The molecular weights of the reference proteins are given in Methods.

In both cases, two bands were separated corresponding to molecular weight values of 134 000 and 62 000, respectively.

This latter result, taken in conjunction with the previously reported formation of two peaks by ultracentrifugation of reduced enterokinase, is consistent with the presence in porcine enterokinase of two chains linked by disulfide bridges; these will be designated heavy (H) and light (L) chains, respectively. The fair agreement between the sum of the molecular weight of the chains and that of the entire enzyme is noteworthy.

Electrophoresis in the presence or absence of β-mercaptoethanol was also applied to labeled [^{32}P]DP-enterokinase, the results are illustrated in Fig. 5. With the unreduced enzyme, all the radioactivity was found under the single band corresponding to the intact molecule. After reduction, it was observed to be exclusively associated with the faster-migrating band related to the light chain. Therefore, it could be concluded that the essential serine of enterokinase is located in the light chain.

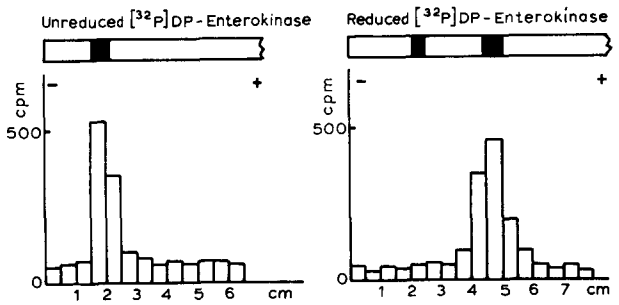

Fig. 5. Electrophoresis of [^{32}P]DP-enterokinase in the absence (left) or presence (right) of 1% β-mercaptoethanol. The position of the bands after coloration with Coomassie Blue is indicated in the upper part of the diagram. The histograms in the lower part indicate the radioactivity measured in successive 0.5-cm-long gel slices after electrophoresis.

Gel filtration of enterokinase and related chains in a dissociating medium

The method first described by Tanford[13] for the determination of molecular weight by filtration through Sepharose 4B in the presence of 6 M guanidine was applied to [^{32}P]DP-enterokinase, to the reference proteins and to their S-carboxymethylated derivatives. The previously identified H and L chains easily separated in this system because of their widely different molecular weights.

Fig. 6 shows that, in sharp contrast with the previously described electrophoresis assays, gel filtration in the presence of 6 M guanidine discriminates between the reduced and unreduced forms of the reference proteins. Values of 210 000, 125 000 and 50 000 were found for the molecular weight of intact enterokinase heavy chain and light chains, respectively. When 6 M guanidine was replaced by 0.1% dodecyl sulfate, two calibration curves were again obtained and the observed molecular weight values were 200 000, 142 000 and 55 000. The position of the essential serine in the light chain could be confirmed in both cases by radioactivity determination.

Finally, the stable S-carboxymethylated derivatives of the H and L chains were obtained at the preparative scale by gel filtration in the presence of 0.1%

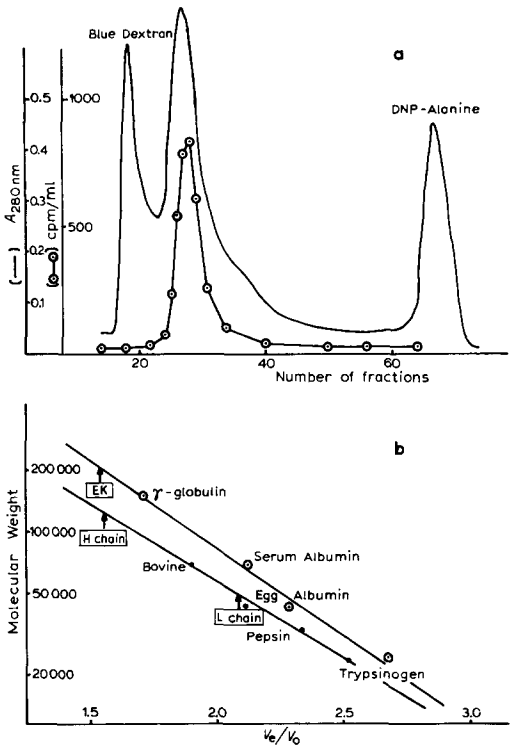

Fig. 6. Filtration through Sepharose 4B in a 6 M guanidine·HCl–HCl buffer (pH 7.2). The behavior of blue dextran, [^{32}P]DP-enterokinase and dinitrophenyl (DNP)-alanine in this system is shown in the upper diagram. In the lower diagram, the elution volume:void volume ratios (V_e/V_0) are plotted against the logarithm of the molecular weight of the unreduced (○) or reduced (●) proteins. The molecular weight of enterokinase (EK) is calculated by reference to the intact protein curve, that of the H and L chains is derived from the curve related to the S-CM-proteins.

sodium dodecyl sulfate. In a typical assay, 100 mg of S-CM-enterokinase were dissolved in 5 ml of a 10 mM sodium phosphate buffer (pH 7.2) 0.1% in sodium dodecyl sulfate. Passage of the solution through a 2.5 cm × 200 cm Sephadex G-200 column equilibrated with the same buffer led to a clean separation of the unretarded H chain from the slower-migrating L chain. The corresponding fractions were pooled, exhaustively dialyzed for 3 days against frequent changes of water and lyophilized.

The values derived from all these assays for the molecular weight of enterokinase and related chains are listed in Table II. In spite of the unusually high sugar content of enterokinase which might have been expected to cause some difficulties, an excellent agreement is seen to exist between the data. These data were not averaged because of the different degree of accuracy and reproducibility of the techniques used. The most likely values are considered to be those given by gel electrophoresis in the presence of sodium dodecyl sulfate (195 000 for enterokinase; 134 000 and 62 000 for the H and L chains, respectively).

Amino acid and sugar composition

The amino acid composition of enterokinase and related chains is summarized

TABLE II

MOLECULAR WEIGHT OF ENTEROKINASE AND CHAINS

Method used	*Enterokinase*	*Chains*		
		Heavy	*Light*	*Sum*
Ultracentrifugation	194 000	–	–	–
Sephadex G-200	212 000	–	–	–
Gel electrophoresis (sodium dodecyl sulfate)	195 000	134 000	62 000	196 000
Sepharose 4B (guanidine)	210 000	125 000	55 000	180 000
Sepharose 4B (sodium dodecyl sulfate)	200 000	142 000	55 000	197 000
Amino acid analysis	197 000	143 000	62 000	205 000

TABLE III

AMINO ACIDS AND OSAMINES IN ENTEROKINASE AND CHAINS

	Enterokinase (residues/mole)	*Chains (residues/mole)*		
		Heavy	*Light*	*Sum*
Ala	63.9	40.0	26.9	66.9
Arg	29.5	17.1	16.2	33.3
Asx	130.2	93.9	36.9	130.8
1/2 Cys[a]	44.9	12.9	4.9	17.8
Glx	124.4	82.2	41.8	124.0
Gly	86.2	55.0	31.6	86.6
His	18.5	9.0	7.1	16.1
Ile[b]	56.1	36.2	20.0	56.2
Leu[b]	91.3	58.1	30.8	88.9
Lys	39.8	26.1	15.4	41.5
Met[c]	16.0	7.0	5.0	12.0
Phe	54.3	35.9	11.3	47.2
Pro	66.5	43.6	21.5	65.1
Ser[d]	84.4	57.9	23.9	81.8
Thr[d]	72.2	52.7	17.8	70.5
Trp[e]	12.0	—	—	—
Tyr	36.2	21.1	11.7	32.8
Val	65.0	43.6	22.9	66.5
Total No. of residues	1091	692	346	1038
Weight of residues	127 264	76 687	37 814	114 501
Total in percents of dry weight	65	57	61	58
Sugars (%)	35	43	39	42
Glucosamine[f]	147.0	100.0	30.8	131.0
Galactosamine[f]	47.9	46.6	26.2	62.8

[a] As cysteic acid in enterokinase after performic acid oxidation of the protein; as S-CM-cysteine in the H and L chains (see text).

[b] The highest value after 72 h hydrolysis was taken.

[c] As methionine sulfone in enterokinase after performic acid oxidation; as methionine in the chains.

[d] After extrapolation to zero time of the results obtained after 24, 48 and 72 h hydrolysis.

[e] Spectrophotometry on the intact protein.

[f] After extrapolation to zero time of the results obtained after 6, 15 and 24 h hydrolysis.

in Table III. Figures in the table indicate the number of residues per mole of protein or chain. The molecular weight values adopted in this case are those derived from the results of the entire analysis by the method of Delaage *et al.*[18] (197 000, 143 000 and 62 000; see Table II, Amino acid analysis).

An excellent or fair agreement can be noted in Table III between the sum of the residues in the two chains and those present in intact enterokinase, with the notable exception of the sulfur-containing amino acids, cystine and methionine which are much lower in the chains. The origin of this descrepancy will be discussed later.

Finally, estimations of sugars by the techniques reported in Methods gave for intact enterokinase: neutral sugars: 20%; amino sugars: 15% and sialic acids: 2% (total, 37%). Corresponding data for the H and L chains are not yet available.

DISCUSSION

Enterokinase plays an essential role for the *in vivo* activation of the pancreatic zymogens. The fact that it is normally bound to a highly specialized membrane (the duodenal brush-border membrane[14]) and that it is a glycoprotein with an unusually high sugar content (about 40%), prompted us to study some of its chemical parameters. This type of study is known to require much more material than those devoted to the catalytic properties of an enzyme. Our first concern, therefore, was to scale up the purification technique worked out 2 years ago in the laboratory for specificity investigations[6]. With the new technique, it was possible to prepare, in each operation starting with 5 kg of porcine mucosa (20 kg of duodenum), about 75 mg (0.4 μmole) of the pure enzyme completely free of aminopeptidase activity. It is clear, however, that this method can still be improved by including, for instance, an affinity chromatography step. It is also clear that the full purification of enterokinase will always remain a laborious task because the enzyme level in the mucosa is quite low. It is of interest to stress in this respect that homogeneity during the preparation was only attained after a 1000-fold purification, calculated from the first pH 6.0 supernatant.

Much time and effort was devoted to the determination of the molecular weight of enterokinase, partly because this enzyme is, as has already been reported, a glycoprotein with a high sugar content. An interesting point was to see whether the presence of the sugars affected the responses of one or several of the techniques employed. As a matter of fact, 6 very different experimental approaches (*i.e.* equilibrium ultracentrifugation, gel electrophoresis in the presence of dodecyl sulfate, gel filtration in a dissociating or non-dissociating medium and amino acid analysis) all led to results diverging by not more than 8% (extreme values, 193 000–213 000). For ultracentrifugation, however, serious errors would have been made if the partial specific volume of the molecule had not been experimentally measured. The relatively low figure found for this parameter (0.705 g/ml) is indicative of a rather loose structure of the molecule, perhaps due to water penetration into the hydrophilic sugar network.

Similarly, the substantial agreement between the results given by ultracentrifugation and Sephadex filtration in the absence of dissociating and reducing agents suggests that, in spite of the presence of the bulky sugar part, the shape and Stoke's radius of the enterokinase molecule are roughly similar to those of the refer-

ence proteins used for column calibration. It is probably significant in this respect that, as discussed later in more detail, the sugars appear to be uniformly distributed throughout the enzyme molecule. Another glycoprotein in which the sugar part is more clearly separated from the polypeptide backbone would perhaps not lead to the same results.

It was also remarkable that correct values for the molecular weight of intact enterokinase could be derived from gel electrophoresis and filtration assays in which unreduced enzyme and reference proteins were used. It can be concluded, therefore, that these techniques, until now mostly employed with reduced proteins and their S-carboxymethylated derivatives, may be applied, at least in some cases, to unreduced molecules. Under these conditions, the accuracy was found to be still largely sufficient to decide whether or not a protein contains non-covalently linked subunits, as reported here.

It must also be emphasized that identical molecular weight values were obtained for enterokinase by gel filtration in the presence of 6 M guanidine or 1% sodium dodecyl sulfate. Sodium dodecyl sulfate has been reported to form complete rod-like structures with proteins, whereas guanidine converts them into random coils[18,19].

It was somewhat unexpected that an enzyme with a molecular weight as high as 195 000 (127 000 for the polypeptide backbone alone) did not dissociate into subunits under the influence of 6 M guanidine or 1% dodecyl sulfate. This enzyme, however, could be shown to be formed by two chains of unequal length, the heavy and the light chain, linked in the intact molecule by disulfide bridges. The light chain was observed to contain the serine residue involved in the catalytic site. The position of the essential histidine is under investigation. Enterokinase is probably the first intestinal enzyme whose catalytic site is demonstrated to be under the control of a serine and a histidine residue.

Sugars have been repeatedly reported to induce partial degradation of a number of amino acids during acid hydrolysis. No serious difficulties were encountered here, except for the sulfur-containing cystine and methionine, which were found in much lower amounts in the chains than in the intact enzyme. The cause of this low recovery cannot yet be discerned. However, it should be mentioned that the above-cited amino acids have been determined as cysteic acid and methionine sulfone in performic-oxidized enterokinase, but were determined as CM-cysteine and methionine in the chains. Tryptophan has not yet been measured.

Notwithstanding these present uncertainties, Table III clearly shows that enterokinase approximately contains 1100 residues, with about 700 in the heavy chain and 350–400 in the light chain. These residues account for about 60–65% of the dry weight of the protein which, consequently, should contain 35–40% of sugars. An important point already stressed before is that the sugar content of both chains appears to be similar.

Table III also shows that the amino acid composition of enterokinase reveals no special features, except for a low content of aromatic residues (tryptophan, 1.1% of the polypeptide part of the molecule; tyrosine, 3.3%) leading to a relatively low extinction coefficient ($E^{1\%}_{1cm} = 8.6$). The content of aspartyl and glutamyl residues (24%) appears to be relatively high. Most of these residues must be in the acidic form since the isoelectric point of enterokinase determined by electrofocusing is 4.2. More-

over, the polar:apolar residue ratio calculated according to Hatch[21] is 1.38. Values ranging from 1.55 for the freely soluble pancreatic enzymes to 1.28 for the proteins of the mitochondrial membrane[21] have been found. These latter proteins have been reported to possess a compact structure and to easily aggregate in aqueous solution.

Finally, the data obtained for the sugar content of enterokinase, either by direct determination (37%) or by difference between molecular weight and total weight of amino acid residues (35–40%) are very similar. They fully confirm the earlier value of 40% reported a number of years ago by Yamashina[4]. Although their role in enterokinase function is still unknown, these sugars may provisionally be assumed to be more important for the binding of the enzyme to the brush-border membrane and/or for its passage through the membrane, than for catalysis itself.

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