The Purification and Characterization of Bovine Enterokinase from Membrane Fragments in the Duodenal Mucosal Fluid*

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Bovine enterokinase has been purified from the mucosal fluid adhering to the intestinal wall. Enterokinase is predominantly present as membrane fragments which must be treated with Triton X-100 to release the enzyme. The purification resulted in a higher yield of enzyme in fewer steps and in less time than when mucosal cells were used. The properties of the enzyme in the fluid are identical with those found previously with the mucosal cell preparation (Liepnieks, J. J., and Light, A. (1979) J. Biol. Chem. 254, 1677–1683), but differ in the size of the subunits and in amino acid composition from the enzyme purified from intestinal contents (Anderson, L. E. Walsh, K. A., and Neurath, H. (1977) Biochemistry 16, 3354–3360). It is highly unlikely that the existence of isoenzymes could explain these differences. It is more likely that the enzyme isolated from the intestinal contents represents an extensively degraded form with retention of enzymatic activity.

Enterokinase (enteropeptidase, EC 3.4.21.9) activates pancreatic trypsinogen and initiates the intestinal digestion of proteins (1, 2). The enzyme is found in the duodenal brush-border membrane and the intestinal contents. Kuntiz (3) was the first to partially purify the porcine enzyme from the intestinal contents. Maroux et al. (2) developed a procedure for the preparation of a highly purified sample and also isolated the enzyme from a detergent extract of the duodenal mucosa (5). The mucosa was chosen as the source for the purification because it contained greater amounts of the enzyme.

More recently, the bovine enzyme was purified from intestinal contents (6) and from mucosal cells (7). Although the two preparations had many properties in common, they showed several significant differences. The mucosal and the intestinal content enzymes differed in the size of the heavy chain (115,000 and 82,000 daltons) and light chain (35,000 and 57,000 daltons). The amino acid compositions also differed.

In an attempt to better understand the relationship between the two forms of the bovine enzyme, we considered the possibility that isoenzymes exist in the cow (7). In our earlier studies, we examined all fractions for enterokinase activity in the course of purification from mucosa, but failed to find a second form of the enzyme. In the hope that we could resolve the differences, we have now examined the properties of the enzyme in the viscous fluid adhering to the lining of the intestinal wall. We selected this source because it contains the enzyme soon after release from the microvilli and reasoned that it could contain a form of the enzyme that is not membrane-bound and that could differ from the mucosal cell preparation. These studies of multiple forms of the enzyme are important in light of the species differences that have been found. For example, the molecular weight of the enzyme from the cow is approximately 150,000 (6, 7), the pig 195,000 (5), and the human 300,000 (8). One would have expected greater similarities since the enzyme performs the same physiological function in these species. The studies described below show that the enterokinase molecule from the mucosal fluid is identical with that found previously from the mucosal cells but different from that in the intestinal contents.

EXPERIMENTAL PROCEDURES AND RESULTS

The approach taken in these studies was to characterize enterokinase from the intestinal mucosal fluid to see if the enzyme is identical with or related to the preparation obtained from the intestinal mucosal cell (7). The properties of the purified mucosal fluid enterokinase were identical with those found from the intestinal wall. We selected this source because it contains the two forms of the bovine enzyme, we considered the possibility that isoenzymes exist in the cow (7). In our earlier studies, we examined all fractions for enterokinase activity in the course of purification from mucosa, but failed to find a second form of the enzyme. In the hope that we could resolve the differences, we have now examined the properties of the enzyme in mucosal fluid adhering to the lining of the intestinal wall. We selected this source because it contains the enzyme soon after release from the microvilli and reasoned that it could contain a form of the enzyme that is not membrane-bound and that could differ from the mucosal cell preparation. These studies of multiple forms of the enzyme are important in light of the species differences that have been found. For example, the molecular weight of the enzyme from the cow is approximately 150,000 (6, 7), the pig 195,000 (5), and the human 300,000 (8). One would have expected greater similarities since the enzyme performs the same physiological function in these species. The studies described below show that the enterokinase molecule from the mucosal fluid is identical with that found previously from the mucosal cells but different from that in the intestinal contents.

DISCUSSION

The identical size of the heavy and light chains of the two enzyme preparations eliminates a structural modification on release from the brush-border membrane. It is known that

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* Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–6, and Tables I and II) are presented in miniprint at the end of this paper. The abbreviations used are: umbelliferyl-p-guanidinobenzoate; Tos-Arg-OMe, N°-tosyl-L-arginine methyl ester; Br-Arg-OEt, N°-benzoyl-L-arginine ethyl ester; Tos-Lys-OMe, N°-tosyl-L-lysine methyl ester; Gdn·Be·ONp, p-nitrophenyl-p°·guanidineboronate; PAS, periodic acid-Schiff reagent; PTI, basic pancreatic inhibitor (Kunitz); SDS, sodium dodecyl sulfate. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-2159, cite the authors, and include a check or money order for $6.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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the enzyme is released from brush-border membranes by limited proteolysis with a loss of the membrane anchor (18, 39-41). The mucosal fluid enterokinase has a polypeptide and carboxylate anchor, since the enzyme is incorporated into reconstituted phospholipid vesicles. We concluded that the desquamation anchor, since the enzyme is incorporated into reconstituted brush-border membranes by proteolysis of the tips of the brush-border membrane microvilli releases the intact enzyme as membrane fragments into the mucosal fluid. Only later, when the enterokinase enters the luminal contents, will the phospholipid-enzyme complex dissociate, releasing the free enzyme.

The identical properties of the mucosal fluid and the mucosal cell enterokinase decrease the possibility of finding an isoenzyme. It seems reasonable that the enzyme isolated by Anderson et al. (6) from the intestinal contents represents a molecule altered by limited proteolysis and/or by degradation of the polysaccharide chains. Proteolysis in the intestinal contents is highly likely since the digestive enzymes are present in large amounts. Various enzymes that degrade carbohydrates are also present. Since both the heavy and light chains of enterokinase contain approximately 35% carbohydrate, both chains could be modified.

Finally, the activation peptide sequence of trypsinogen is highly conserved in different species. The sequences of Asp-Lys found in most species of the Glu-Asp-Lys and Glu-Asp-Lys of lungfish trypsinogen all have a cluster of acidic residues preceding the lysine (42-44). The substrate specificity of enterokinase must recognize these positions (2, 45), because the enzyme has a high catalytic efficiency toward these sequences relative to the low efficiency of bovine trypsin. Thus, the structure of enterokinase, at least in the region of the substrate binding and active site residues, must also be conserved and a physiological function for isoenzymes does not appear likely.

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**Bovine Enterokinase**

**EXPERIMENTAL PROCEDURES AND RESULTS**

**MATERIALS**

- Bovine trypsin, bovine trypsinogen, and soybean trypsin inhibitor were obtained from Behring Diagnostics, Inc.
- Results were published in the May 1971 issue of the Amer. J. Physiol.
- The enzyme activity was determined using the method of Oyanagi and Kozasa (28).

**METHOD**

**Purification of Enterokinase**

- From bovine duodenal brush or digestor fluid, 5 g of a 60 mM sucrose, 0.01 M Tris-HCl, pH 8.0, was homogenized with 30 g of Sabouraud's dextrose agar medium. The homogenate was filtered to remove larger particles. The enzyme activity was determined using the method of Oyanagi and Kozasa (28).

**RESULTS**

**Properties of Enterokinase**

- The level of enterokinase activity in the intestinal fluid was measured in the presence of the enterokinase and 0.1 M NaCl. The activity was measured in the presence of the enterokinase and 0.1 M NaCl.

- The presence of bile salts and proteases in the intestinal fluid could be responsible for the release of enterokinase from the intestinal fluid. Bile has been shown to release enterokinase from the intestinal epithelial cells. The presence of enterokinase in the intestinal fluid has been demonstrated by Oyanagi and Kozasa (28).

**SUPPLEMENTARY MATERIAL**

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Bovine Enterokinase

(37) also reported a requirement for calcium in the activation of trypsinogen with porcine enterokinase. This requirement may be a result of calcium ions binding to the apoprotein residues of the activation peptide of trypsinogen and also increasing the stability of both trypsinogen and the product trypsin (38).

The purified enzyme was reconstituted into soybean phospholipid vesicles with 14.8% of the soybean phospholipids bound in the membrane bilayer. The vesicle fluid enterokinase behaved in the same way as the vesicle fluid in the presence of calcium (38). In a previous study, enterokinase released from the brush border membrane by papain could not associate with the vesicles presumably because the apoprotein was left in the membrane (38). Apparently, the enzyme obtained from the vesicle fluid contained the same region of the polypeptide chain responsible for anchoring enterokinase to the membrane.

Fig. 1. Sucrose gradient centrifugation of the homogenate of mucosal fluid. 200 μl of a 1× homogenate (v/v) in 50 mM mannitol, 2 mM Tris-HCl, pH 7.1, were layered above a 9 to 68% sucrose gradient in mannitol buffer. Centrifuged 12 h at 200,000 g, 20°C. 0.1 ml fractions were collected. Fraction 1 is from the bottom of the tube. Sedimentation of unbound enzyme was performed in a separate experiment. Enzyme activity per fraction (O--O); unbound enterokinase units per fraction (C--C).

Fig. 2. Gel filtration chromatography of the homogenate of the mucosal fluid. 1 ml of the homogenate (1× w/v) in 50 mM mannitol, 2 mM Tris-HCl, pH 7.1, was applied to Sepharose 6-1000 1.75 × 30 cm. One ml fractions were collected at a flow rate of 10 ml/hr. 45° Entero kinase activity per fraction (O--O); phosphorus per fraction (C--C).

Fig. 3. SDS-polyacrylamide gel (15% w/v) electrophoresis of mucosal cell or mucosal fluid enterokinase. A) mucosal fluid enterokinase in the absence of aprotinin; B) mucosal cell enterokinase in the absence of aprotinin; C) mucosal fluid enterokinase in the presence of aprotinin; D) mucosal cell enterokinase in the presence of aprotinin.

Fig. 4. Isolelectric focusing of mucosal cell and mucosal fluid enterokinase. A) mucosal fluid enterokinase; B) mucosal cell enterokinase. Gel was equilibrated with ampholines, pH 3-10. Molecularity: B, pH 3.20; V, aprotinin; H, pH 4.95, soybean trypsin inhibitor; and pH 6.85, glacial acetic acid.

Fig. 5. Inhibition of enterokinase by PPI. Mucosal fluid or mucosal cell enterokinase (25 μg) and PPI (1 to 50 mM) were incubated at 37°C. After 20 min, at room temperature. Residual activity was determined by titration with gelatin. Mucosal fluid enterokinase (O--O); mucosal cell enterokinase (C--C).
Bovine Enterokinase

Fig. 6. Reaction of mucosal fluid, mucosal cell enterokinase, and bovine trypsin with Gdn-Bz-OMud. Increase of fluorescence with time followed after addition of 1 ml of Gdn-Bz-OMud to 0.05 ml of enterokinase or 0.06 ml of trypsin in 3.0 ml of 0.1 M Veronal, 2 M CaCl₂, pH 8.3. See text for details. Mucosal fluid enterokinase (___); mucosal cell enterokinase (---); bovine trypsin (----).

Table I

| Source          | Total Activity (mg) | Specific Activity (units/mg) |
|-----------------|--------------------|-----------------------------|
| Enterokinase    | 3.2 x 10⁴          | 2                            |
| Mucosal fluid   | 1.8 x 10⁵          | 170                          |
| Enterokinase    | 3.4 x 10⁴          | 2                            |
| Mucosal fluid   | 1.6 x 10⁵          | 170                          |
| Enterokinase    | 3.2 x 10⁴          | 2                            |
| Mucosal fluid   | 1.8 x 10⁵          | 170                          |

**Note:** 100 ml of either mucosal cells or mucosal fluid (pig weight) was homogenized in 340 ml ice-cold 30 mM mannitol, 1 mM Tris-Cl pH 7.1, 2 mM, 4°C.

Table II

| Purification Step | Total Activity (mg) | Specific Activity (units/mg) | Purification Fold |
|------------------|--------------------|-----------------------------|-------------------|
| 1% Triton X-100 extract | 4.9 x 10⁴         | 2                           | 716               |
| 70% saturation (NH₄)₂SO₄ precipitate | 4.3 x 10⁷         | 160                         | 89                |
| pH 5.0 supernatant | 3.9 x 10⁷         | 750                         | 1000              |
| DEAE-cellulose chromatography | 2.3 x 10⁶         | 2000                        | 1000              |
| Protein determined spectrophotometrically. |                  |                             |                   |

**Note:** Proteins determined by the method of Lowry et al. (21).
The purification and characterization of bovine enterokinase from membrane
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P Fonseca and A Light

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