Characterisation of a novel proteolytic enzyme localised to goblet cells in rat and man

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SUMMARY A proteolytic enzyme, ingobsin, purified from rat duodenal extracts is shown to be localised to intestinal goblet cells of both man and rat. Enzyme positive cells decrease in number from duodenum to colon. The enzyme is a 33 000 M protein with an isoelectric point of 5.1. The pH optimum for enzymatic activity is 7.4–8.0. Based on substrate specificity for arg-x, lys-x and to a lesser degree tyr-x, on the effect of diisopropylphosphorofluoride, Trasylol and phenylmethylsulfonylfluoride and on proteolytic activity towards intact proteins, ingobsin is classified as a serine proteinase with endoproteolytic activity.

Large amounts of goblet cells are localised in the surface epithelium of the intestinal mucosa. Their main function is the production of mucus which is of importance for a normal intestinal function.1

During the isolation of duodenal epidermal growth factor2 we concomitantly purified an enzyme that proved to be localised to the goblet cells. The present paper presents data on the purification, characterisation, and localisation of this hitherto unknown enzyme.

Methods

MATERIALS
Purification procedure
Duodena were removed from Wistar rats under ether anaesthesia and kept frozen until used. For a typical purification procedure 25 duodena – that is, approximately 25 g tissue – were homogenised in 100 ml acetic acid, 50 mmol/l. After freeze thawing pH was adjusted to 4.5 and the solution was centrifuged 10 minutes, 20 000 rpm at 4°C. The precipitate was washed twice with 50 ml acetic acid, 50 mmol/l. The combined supernatants were filtered through glasswool, lyophilised, and redissolved in 70 ml hydrochloric acid 50 mmol/l, sodium chloride 0.15 mol/l, pH 1.5. After centrifugation for 30 minutes, 20 000 rpm at 4°C, the supernatant was lyophilised, redissolved in 8 ml distilled water, and pH was adjusted to 1.5. The sample was prepared on a 20×1100 mm P10 Biogel (BioRad, Richmond, California, USA) column run at 14.5 ml/h with hydrochloric acid 50 mmol/l, sodium chloride 0.15 mol/l, pH 1.5. Fractions of 2.9 ml were collected. The enzyme containing fractions were pooled, dialysed against distilled water, lyophilised, redissolved in 3.5 ml imidazole 0.025 mmol/l chromatofocusing buffer (Pharmacia A/S, Uppsala, Sweden), and pH adjusted to 7.4. The sample was subjected to chromatofocusing on a 9×135 mm (8 ml) column (Pharmacia A/S) and run with a pH gradient from 7.4 to 4. The flow rate was 26 ml/h and fractions of 1 ml were collected. The enzyme containing fractions were pooled, dialysed, lyophilised, and redissolved in 1 ml of water. pH was adjusted to 8.0, and the sample was filtered through a 10×450 mm G-75 Sephadex (Pharmacia A/S, Uppsala, Sweden) column run with ammonium bicarbonate 1 mol/l, pH 8.0, at a flow rate of 5.5 ml/h. Fractions of 1.1 ml were collected. The enzyme containing fractions were lyophilised and kept at −20°C until used for further studies.

Production of antibody
A rabbit was given approximately 30 µg (1 nmol) enzyme (300 µl) mixed with an equal volume of Freund's complete adjuvant (Behringwerke, Marburg, FRG) by multiple intradermal injections on the back. Three booster injections were given at two week intervals by injecting approximately 30 µg (1 nmol) of enzyme mixed with incomplete Freund's adjuvant (Behringwerke, Marburg, FRG). Antiserum was obtained by aortic puncture.
**Immunohistochemical studies**

Rat duodenum, jejenum, ileum, colon, submandibular gland, trachea, pancreas, liver, kidney, and stomach were obtained from Wistar rats. A human duodenal specimen was obtained from a patient undergoing Billroth I partial gastrectomy. The tissues were fixed at room temperature in Buin’s fixative by immersion for 24 hours, dehydrated, and embedded in paraffin.

The enzyme was localised by the unlabelled antibody peroxidaseantiperoxidase (PAP) technique. The primary antiserum was used at dilutions of 1/100 and 1/400. In controls, the primary specific antibody was replaced by non-immune rabbit serum or by antiserum preincubated with the enzyme.

**Stokes radius**

Stokes radius was calculated based on data obtained by G-200 Sephadex (Pharmacia A/S), Dextran blue and $^{22}$Na were used as markers for void and total volume. Human albumin was used as internal calibration (Stokes radius 3.6 nm).

**Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis**

Fifteen or 12 1/2% continuous gels or 9–20% discontinuous gels were run as described on samples reduced by heating to 100°C in the presence of dithiothreitol.

Iodinated samples were exposed for 24 hours. Gels with $^3$HDFP labelled samples (see below) were incubated with Enhance NEF 966 (NEN, Boston, USA) and exposed for two weeks at −20°C. The plates used were Kodak XL-1 (Roskilde, DK).

Immunoblotting of the separated polypeptides was performed as described on nitrocellulose AAHY 304 FO (Millipore, Bedford, USA). The protein bands were visualised by incubation with antibody or non-immune serum 1:100 overnight after two hours incubation with peroxidase labelled swine anti-rabbit immunoglobulin 1:1000 (DAKO, Copenhagen, DK). A colour reaction was developed by approximately 10 minutes incubation with 3-amino-9-ethyl-carbazole (Sigma, St Louis, USA).

**Isoelectric focusing**

Isoelectric focusing was carried out as described by the manufacturer on a LKB equipment (Bromma, Sweden) using a pH gradient from 3.5 to 9.5. The gel was cut into 5 mm pieces, the enzyme was eluted overnight in 500 μl phosphate buffer and tested as described below.

**Amino acid analysis**

Amino acid analysis was performed on 15 μg protein hydrolysed for 24 hours using a Durrum D 500 amino acid analyser. Reagents used for this procedure were from Dionex (Sunnyvale, California, USA) analytical grade.

**Test for enzymatic activity**

The enzyme cleaved $^{125}$I-EGF so that it could no longer be precipitated by trichloroacetic acid. Approximately 100 000 cpm $^{125}$I mouse EGF was incubated for one hour at room temperature with 100 μl sample, mixed with 300 μl buffer and precipitated with 1 ml 20% trichloroacetic acid (v/v) for 10 minutes on ice. After centrifugation for 20 minutes at 3000 rpm, the enzyme activity was expressed as the fraction of $^{125}$I remaining in the supernatant. The buffer was phosphate 0.1 mol/l, sodium chloride 0.15 mol/l, 0.1% human albumin (Behringwerke, Marburg, FRG), pH 7.4. For quantification of the enzyme, a calibration curve ranging from 0.05 to 1 μg/ml (1.5–33 nmol/l) purified enzyme dissolved in testbuffer was included. Buffers for determination of the pH optimum were hydrochloric acid or phosphate buffers containing sodium chloride 0.15 mol/l and 0.1% albumin (Behringwerke).

**Enzymatic activity towards synthetic substrates**

This was determined essentially as described. In brief, 10 μl (1 μg) (33 pmol) enzyme was added to 500 μl substrate, 0.25 mmol/l dissolved in buffer (sodium phosphate 0.1 mol/l, EDTA 75 μmol/l, pH 8.0). The change in absorbance at 246, 250, or 255 nm, depending on the substrate, was recorded on a Unicam spectrophotometer.

The following substrates and enzymes were obtained from Sigma (St Louis, USA): N-alpha-benzoyl-L-alanine-methylester, N-alpha-benzoyl-L-arginine-ethylester, N-benzoyl-L-tyrosine-ethylester, N-alpha-p-tosyl-arginine-methylester, N-alpha-p-tosyl-L-lysine-methylester, N-benzoyl-glycine-arginine, alpha-chymotrypsin (EC 3.4.21.1), elastase (EC 3.4.21.11), and phenylmethylsulfonylfluoride. Trasylol 20 000 KIU/ml was from Bayer (Leverkusen, FRG). Trypsin TPCK (EC 3.4.21.4) was obtained from Worthington (Freehold, New Jersey, USA) and submandibular protease was obtained from Boehringer Mannheim, (FRG).

**Iodination**

This was performed using the chloramin T method. In brief, approximately 0.2 nmol (6 μg) of protein dissolved in 200 μl 0.2 mol/l phosphate buffer, pH 7.6, was iodinated with 1 μCi $^{125}$I (Amersham, UK) by vigorous mixing with 20 μl chloramin T (0.25 mg/ml) (Merck, USA) for 40 seconds. Iodination was terminated by addition of 20 μl sodium hydroxide.
bisulphite (Merck, USA) (0.5 mg/ml) and vigorous mixing for 15 seconds. After addition of 1 ml 0.1 mol/l phosphate, 0.15 mol/l sodium chloride, 0.1% albumin free and protein bound iodine was separated by dialysis against phosphate buffer overnight or by gel filtration on G-75 Sephadex as described above.

**Labelling of enzyme**

Labelling of enzyme with (1,3-H)3 Di-isopropyl phosphorofluoridate, 6 Ci/mmole (3HDFP) (Amersham, UK). Approximately 33 µg enzyme (1 nmol) dissolved in 500 µM 0.1 mol/l phosphate, 0.15 mol/l sodium chloride, 0.1% albumin, pH 8, was incubated with 20 nmol 3HDFP for eight hours at room temperature and at 4°C overnight, in the presence or absence of 50 KIU Trasylol. As a positive control, bovine trypsin approximately 2 nmol was labelled in a similar manner.

**Digestion of native proteins**

This was assessed using human albumin (Behringwerke, Marburg, FRG), and three cobalamin binding proteins purified as previously described10 (human intrinsic factor, rabbit transcobalamin and human haptocorrin). Approximately 40 µg of protein (0.7-1 nmol) dissolved in 50 µl ammonium bicarbonate 0.1 mol/l, pH 8.0, was digested with 0.4 µg (12 pmol) enzyme for 18 hours at 37°C. For binding studies, 0.25 pmol of haptocorrin or intrinsic factor was mixed with approximately equal amounts of 57Co-cyanocobalamin (Amersham, UK) adjusted to a specific activity of 0.1 µCi/pmol with cyanocobalamin (Dumex, Copenhagen, DK). After incubation overnight at room temperature aliquots of 50 µl were incubated for three hours at room temperature with 25 µl of the test solution. The buffer was sodium phosphate 0.1 mol/l, sodium chloride 0.15 mol/l, human albumin 0.1%, pH 8.0. In a second set of experiments, the enzymatic digestion was performed before incubation with the labelled cobalamin. Free and protein bound cobalamin were separated by charcoal precipitation.11 To compare digestion of native and heat denatured (100°C for 10 minutes) protein, haptocorrin was iodinated as described above. Digestion 125I haptocorrin, of 125I insulin (Novo, Copenhagen, DK), and 125I EGF was performed as described for the cobalamin labelled proteins. Digested peptide (protein) was estimated by calculation of the amounts of 125I remaining in the supernatant after precipitation with trichloroacetic acid in a final concentration of 13%.

**Concentration of proteins**

This was estimated by the method of Lowry.12 Human albumin (Behringwerke, Marburg, FRG) was used as calibrator.

**Immunoprecipitation of enzyme**

This was performed using swine anti-rabbit gammaglobulin (DAKO, Copenhagen, DK). Thirty three picomoles (1 µg) enzyme was mixed with 10 µl antiserum incubated for one hour at room temperature in a total volume of 1 ml phosphate buffer; 100 µl second antibody was then added and the samples were incubated overnight at 4°C. Antibody precipitated material was collected after three times washing with 0.1 mol/l phosphate, 0.15 mol/l sodium chloride, and centrifugation at 20 000 g for 30 minutes between each washing. Control samples were incubated with non-immune serum instead of antiserum.

**Results**

An enzymatic activity from rat duodenal extracts has been purified by combining gel chromatography and chromatofocusing (Fig. 1). Approximately 33 nmol (1 mg) of enzyme could be purified from 50 g of starting material — that is, 50 duodena. This represents a yield of around 20%. The enzyme eluted with a KD of approximately 0-6 at Biogel P10, pH 1-5, and not as expected from its molecular size with a KD close to zero. A similar retention of P10 Biogel has been described and used for the purification of epidermal growth factor.13 Stokes radius estimated by G-200 Sephadex gel filtration of the enzyme was 1-9 nm. In accordance with this SDS-polyacrylamide gel electrophoresis of unlabelled enzyme, iodinated enzyme, or 3HDFP labelled enzyme showed one band with a relative molecular mass of 33 000. The same band was seen by Western blotting (Fig. 2). The much weaker bands sometimes seen by SDS electrophoresis had lower relative molecular masses and varied from preparation to preparation. These are most likely created by autodigestion. Carbohydrates could not be detected by either carbohydrate staining of the gels or by amino acid analysis.

Amino acid analysis of two different preparations gave almost identical results (Table I). It showed a high content of acidic amino acids in accordance with the rather low isoelectric point of 5-1 as determined by isoelectric focusing (Fig. 3). The antibody reacted with the enzyme thought it did not block the enzymatic reactivity. Thus, enzymatic reactivity was recovered in the precipitate, when the enzyme was incubated with antiserum followed by second antibody. No enzymatic reactivity was found when non-immune serum was employed instead of antiserum.
Goblet cell proteolytic enzyme

By immunohistochemical procedures, the enzyme was localised to some of the goblet cells in duodenum from both rat and man (Fig. 4). The reactive cells were concentrated in the epithelium of the crypts and in the lower part of the villi. Their number decreased towards colon, where very few positive cells were detected. Also the cells of the granular convoluted tubules in the submucosal glands of the rat showed a weak positive reaction (data not shown). In contrast all other tissues examined were negative. These include gastric mucosa, tracheal mucosa, pancreas, liver, and kidney.

By enzymatic assay, the enzyme was found in amounts of 50 to 300 μg (1.5–9 nmol) per rat duodenum and from less than 10–100 μg (less than 0.3–3 nmol) in duodenal juice collected over five hours through a catheter placed in a duodenal pouch in a way that prevented contamination with sputum and pancreatic juice.

The purified enzyme was able to digest a number of synthetic substrates as well as native peptides and proteins. The enzymatic activity could be blocked completely by Trasylol (500 KIU/ml) (Fig. 3) and by phenylmethylsulfonylfluoride. The latter blocked 50% of the activity towards benzoyl-arginine-ethylester in a concentration of 2.5 mmol/l. The enzyme also bound ^3^HDFP as seen in Figure 2.

The enzyme proved active towards lys-x, arg-x, and to a lesser degree towards tyr-x. No activity towards ala-x and gly-x was observed (Table 2).

Enzymatic activity towards intact peptides was found for insulin (data not shown) and epidermal growth factor. Though the latter peptide was cleaved so that it could no longer be precipitated by trichloroacetic acid nor be recognised by its receptor, the antigenicity and relative molecular mass as judged by gel filtration was unchanged. These findings suggest the enzyme reacts only on amino acid no 45 (arg-asp) or/and 48 (arg-trp) of this 53 amino acid peptide. The pH optimum for cleavage of epidermal growth factor was 7.4 to 8.0, and the activity of the enzyme towards this peptide was about eight times the activity of trypsin expressed on a molar basis (Fig. 5). Though the enzyme was inactive at pH below 3, the low pH of...
Table 1  Molecular characteristics of rat intestinal goblet cell enzyme, ingobsin, and selected other enzymes

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<th>Ingobsin (rat)*</th>
<th>EGF binding protein (mouse)†</th>
<th>Trypsin (bovine)‡</th>
<th>Chymotrypsin (bovine)‡</th>
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<td>223</td>
<td>245</td>
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| Carbohydrate     | +               | +                            | +                 | +                      | +                     |
| PL               | 5-1             | 5-6                          | 6-7               | 7-2                    | 4-0-4-9               |
| pH optima        | 7.4-8.0         | 7.8-8.5                      | 7.8               | 8.0-9.0                | 5.6-6.2               |
| M<sub>av</sub><sup>-1</sup> | | | | | |
| Localisation     | Intestinal goblet cells | Submandibular gland | Pancreas | Pancreas | Intestinal epithelial cells |

* This study.  Data from: † ref 9, 19; ‡ 25; § 20 and 26.

Discussion

We have purified a proteolytic enzyme from rat duodenum, and suggest the name ingobsin because of its histochemical localisation to intestinal goblet cells of both rat and man. The localisation is surprising, as to our knowledge no proteolytic enzyme has previously been isolated from the goblet cells. Also the distribution along the intestinal tract is curious. Whereas the goblet cells increase in number from duodenum over ileum and jejunum to colon, the number of cells containing enzyme immunoreactivity are most numerous in the duodenum. The enzyme seems localised to young cells, as it is most abundant in the lower part of the villi and in the crypts. This is in contrast with the mucus, which is believed to be secreted throughout the life span of the goblet cells. 16, 17

The enzymatic reactivity of ingobsin for the synthetic substrates lys-x, arg-x, and to a lesser degree tyr-x, combined with the ability of Trasylol and phenylmethylsulfonylfluoride to inhibit the enzymatic activity, and with the ability of <sup>3</sup>HDFP to label to the enzyme shows the enzyme to be a serine proteinase as for example, trypsin, chymotrypsin.
Fig. 2 SDS polyacrylamide gel of the purified enzyme ingobsin. (A) 9–20% gradient gel of reduced samples. Left: marker mix coloured with 0.3% Amidoblack. The markers are from top to bottom: phosphorylase B M, 92 500, albumin M, 67 000, carbonic anhydrase M, 30 000, and alpha-lactalbumin M, 14 400. Middle: 10 µg (0.3 nmol) enzyme seen by immunoblotting with antiserum 1:100. Right: 10 µg (0.3 nmol) enzyme developed by immunoblotting with non-immune serum 1:100. (B, C, D) 12.5% gel of reduced samples. (B) Marker mix (left) and 10 µg (0.3 nmol) enzyme (right) stained with cromassie blue. The markers are from top to bottom: phosphorylase A, M, 94 000 (6 µg); albumin, M, 67 000 (8 µg); ovalbumin, M, 43 000 (15 µg); carbonic anhydrase, M, 30 000 (8 µg); soybean trypsin inhibitor, M, 20 100 (8 µg); and alpha-lactalbumin, M, 14 400 (12 µg). (C) Autoradiogram of approximately 10 000 cpm (left) and 100 000 cpm (right) iodinated purified enzyme. (D) Autoradiogram of 3HDFP labelled enzyme in the presence of Trasylol (left) and in the absence of Trasylol (right). The high molecular band seen in the presence of Trasylol most likely corresponds to a non-specific labelling of albumin. An identical band was seen when labelling trypsin in the presence of Trasylol (data not shown).

Table 2 Enzymatic activity of rat intestinal goblet cell enzyme, ingobsin, and selected other enzymes towards synthetic substrates

<table>
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<th>Substrate</th>
<th>Ingobsin*</th>
<th>EGF binding protein†</th>
<th>Trypsin*</th>
<th>Chymotrypsin*</th>
<th>Elastase*</th>
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Activity is expressed relative to the activity towards tosyl-L-lysine methylester.

* This study. † Data from ref. 9. ‡ Indicates no detectable activity.
the mucus of the goblet cell, is delivered by exocrine secretion.

Ingobsin was discovered because of its ability to cleave the peptide epidermal growth factor even at very low concentrations. Epidermal growth factor is a 53 amino acid polypeptide that among other properties has a protective function on the gastrointestinal surface epithelium. Further, both the intact peptide and the C-terminal shortened 48 amino acid peptide inhibits gastric acid secretion. As ingobsin is most likely to cleave epidermal growth factor at the arg residue in position 48, it seems possible that the enzyme cleaves this mitogenic peptide so that it becomes only a gastric acid inhibitory molecule.

At higher though still physiological concentrations, ingobsin is able to destroy the cobalamin binding capacity of the endogenous glycoprotein haptocorrin, although it does not prevent the binding of cobalamin to intrinsic factor. The normal absorption of vitamin B₁₂ (cobalamin) depends on a transfer of cobalamin, ingested with the food and bound to saliva or gastric haptocorrin, from haptocorrin to the transport protein, intrinsic factor. The physiological mechanism for this is not clarified though it is known that high concentrations of trypsin in vitro does promote the transfer of cobalamin from haptocorrin to intrinsic factor. The present study shows ingobsin to be considerably more potent than trypsin in destroying the
Goblet cell proteolytic enzyme

cobalamin binding capacity of both cobalamin saturated and cobalamin unsaturated haptocorrin.

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