THE PROTEINASES OF HUMAN GASTRIC ADENOCARCINOMATA: THEIR IDENTIFICATION, SEPARATION AND SITES OF ACTION ON THE B-CHAIN OF OXIDIZED INSULIN

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(Received 21 July 1971)

SUMMARY

1. Because of the possibility that the proteinases of gastric adenocarcinomata may differ from those of healthy gastric mucosa, an investigation of these enzymes by means of pH–activity studies, agar-gel electrophoresis, column chromatography and the mode of action on the B-chain of oxidized insulin has been undertaken.

2. Agar-gel electrophoresis of extracts at pH 5 revealed a single zone of proteinase activity situated at the equivalent position to the zone 7 of normal gastric mucosal extracts and not activated at pH 2. The typical pepsins of normal mucosal extracts were not present.

3. Agar-gel electrophoresis at pH 8.2 resolved this single zone into three zones. One was located slightly cathodally (proteinase 1) and the other two anodally (a slower proteinase 2A and a faster 2B).

4. Proteinases 2A and 2B were separated by column chromatography and shown to have identical asymmetrical broad pH–activity curves with the maximum at pH 3.3–3.4.

5. Proteinase 1 had a symmetrical narrow pH–activity curve with the maximum at pH 3.7–3.9. Proteinase 1 was purified and resolved into two highly active major components, 1A and 1B, by column chromatography, first on DEAE-cellulose and then on CM-cellulose.

6. The sites of cleavage of the B-chain of oxidized insulin were determined for proteinases 1A and 1B. The same bonds were split by each, with one exception, but several were split at differing rates indicating that the two enzymes had related, but different, modes of action.

7. The tumour proteinases 1 resemble the cathepsins D in certain respects and the pepsins in others. They may represent an enzyme structure of an intermediate form. There is insufficient evidence to indicate whether they are elaborated by partially differentiated cells or whether they are derived from cells which have become de-differentiated. No enzymes exactly like them have yet been found in normal tissues.

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Extracts of human gastric adenocarcinomata were shown by Taylor (1960a) to digest plasma proteins with a single pH maximum at pH 3.2–3.4. By contrast, the gastric juice of the same patients and extracts of their uninvaded gastric mucosa digested plasma proteins with two pH maxima, at pH 2.1–2.4 and pH 3.3–3.8, thus showing the same proteolytic properties as normal gastric juice and normal gastric mucosal extracts under the same experimental conditions (Taylor, 1959a).

It was noted that the pH maximum for digestion by the tumour extracts was similar in range to the upper maximum shown by normal mucosa, and this similarity was enhanced when it was found that the tumour extracts did not digest egg albumin, which is digested by normal mucosal extracts only at the lower pH maximum.

The original interpretation of these differences between neoplastic and normal tissues was of interest because the double pH maximum is a property of a single pure pepsin (Taylor, 1959b). It was postulated that, with the loss of cell differentiation in the adenocarcinomata, the tumour proteases also reverted to a less specialized or 'primitive' enzyme, which might then be functionally equivalent to the evolutionary precursor of the pepsins. The pepsins would thus have evolved from such a protease by acquiring the ability to digest proteins with a second (lower pH) maximum (Taylor, 1960b). On the other hand, many normal tissues are known to contain cathepsins which are maximally active in vitro at pH 3.4, so that the tumour proteases might be related to these. This could be looked on biologically as a failure of the cells to differentiate rather than as a de-differentiation. There is the possibility that the cathepsins, or one of them, are themselves the less specialized forms from which the pepsins have evolved. The present investigation was therefore undertaken to characterize further the nature of the proteolytic activity that is present in extracts of gastric adenocarcinomata and to throw light on these two hypotheses, which are a biochemical counterpart of a problem which has teased morbid histologists for decades.

A preliminary account of this investigation has been published (Etherington & Taylor, 1969b).

**MATERIALS AND METHODS**

*Preparation of carcinomatous extracts*

Gastric adenocarcinomata were obtained at surgical operation and transported immediately on ice to the laboratory cold room. After thorough washing with cold iso-osmotic NaCl (0.9%, w/v), the centre of the tumour was cut out and freed of as much muscle as possible. This central portion contained adenocarcinomatous cells and invaded muscle cells, but normal mucosal cells were excluded. Extracts were prepared as previously described for mucosal specimens (Etherington & Taylor, 1969a).

*Construction of pH–activity curves*

The method of Anson & Mirsky (1932) was used as modified by Hanley, Boyer & Naughton (1966). Extracts and chromatographic fractions were diluted fivefold with water before adding them to the digestion mixtures, which were then incubated at 37°C for 16 h. The pH was determined from a 4 ml volume of the digestion mixture with a Vibron pH meter (model 39A Electronic Instruments Ltd, Richmond, Surrey).
Agar-gel electrophoresis

The tumour extracts and chromatographic 'fractions' were analysed by electrophoresis in agar gel at pH 5.0 (Etherington & Taylor, 1969a) and at pH 8.2 by the method of Lapresle & Webb (1962). To test for the existence of acid-activated zymogens, extracts were acidified to pH 2.0 and incubated at 37° for 10 min before application to the gels.

Ion-exchange chromatography

DEAE-cellulose (Whatman DE 50) was generated and packed into columns (20 cm x 2.5 cm) (Peterson & Sober, 1962). Extracts were concentrated with Carbowax (20 M grade) to approx. 10 ml, dialysed against 5 mM-potassium phosphate buffer, pH 8.0, and then clarified by centrifuging at 10 000 g for 15 min. The extracts were then applied to the columns, previously equilibrated with the same buffer. Columns were developed with three linear gradients, each of 900 ml total buffer volume. The first gradient was from 5 to 50 mM-potassium phosphate buffer, pH 8.0. The second gradient, which eluted the remaining proteinase activity, was from 0 to 0.5 M-NaCl in the 50 mM-phosphate buffer, pH 8.0. A third gradient to 0.05 M-citric acid ensured that no activity remained adsorbed to the column.

CM-cellulose (Whatman CM 70) was generated and packed into a column (22 cm x 1 cm) (Peterson & Sober, 1962). The proteinase eluted first from DEAE-cellulose was rechromatographed on this column; the appropriate fractions were combined, concentrated by pressure dialysis and dialysed against 10 mM-sodium acetate buffer, pH 5.5, before loading on to the CM-cellulose column, which was equilibrated with the same buffer. The column was developed with a 300 ml linear gradient of 10-50 mM-sodium acetate buffer, pH 5.5, and this was followed with a 300 ml linear gradient to 0.5 M-NaCl in the 50 mM buffer. Activity remaining on the column was eluted by passing through the column in a step-wise manner 40 ml of 50 mM-sodium acetate buffer, pH 5.5, containing 1.0 M-NaCl, followed by 80 ml of 50 mM-potassium phosphate buffer, pH 7.5, containing 1.0 M-NaCl. The bulk of the activity was eluted in this step.

Analysis of effluent fractions

Proteolytic activity was estimated at pH 3.7 and with an incubation time of 16 h (Hanley et al., 1966). The protein concentration was measured from the extinction at 280 nm with an Optica (Gateshead) u.v. spectrophotometer. Effluent fractions were also analysed, where appropriate, for phosphate (Fiske & Subbarow, 1925), chloride by coulometric titration (Evans Electroselenium Ltd, Harlow, U.K.) and pH.

Calculation of enzyme concentration

The amount of proteinase activity was expressed in terms of the equivalent activity of crystalline swine pepsin A (Etherington & Taylor, 1971). Proteinases were buffered at pH 3.7 and compared with known pepsin A concentration which had been incubated at pH 1.9.

Effect of cysteine

Cysteine was incorporated into the digestion mixtures at concentrations of 1.8 mM and 3.6 mM. The extinction of the supernatant obtained after precipitation with trichloroacetic acid was read at 280 nm.
Digestion of the B-chain of oxidized insulin by proteinases from gastric adenocarcinomata

Bovine insulin (Boots, Nottingham, six times recrystallized) was oxidized by the method of Hirs (1956) and the B-chain separated by precipitation (Sanger, 1949). A 5 or 10 mg sample of the B-chain was dissolved in 4 ml of distilled-deionized water and the pH adjusted to near pH 3.5. A predetermined quantity of the tumour proteinase in concentrated solution was added and the pH readjusted to 3.5 with 1 M-HCl delivered from a micrometer syringe (Agla, Burroughs and Wellcome, London). The substrate was digested for 24 h at 37° and then heated at 100° for 5 min to destroy the enzyme (Sanger & Tuppy, 1951). The digestion mixture was freeze-dried and redissolved in 0.25 ml of pyridine-water (1:2, v/v) for the separation and analysis of the constituent peptides (Etherington & Taylor, 1971).

RESULTS

pH–activity curves for whole-tumour extracts

The pH–activity curves for three extracts are shown in Fig. 1. Differences existed between the three curves and these seemed due to the summation of at least two separate types of activity. A fairly narrow peak with a maximum at pH 3.8 was given by two extracts (patients 2 and 3) with a shoulder on the acid side that was almost absent for patient 2. The predominant component in the curve for patient 1 had a maximum at pH 3.3 with the major peak shown by the other two patients possibly located as the shoulder at pH 4.0-4.2.

Agar-gel electrophoresis of whole-tumour extracts

When agar-gel electrophoresis was conducted at pH 5.0, the proteinase activity was found
Proteinases of gastric carcinomata

to remain at the origin, zone 7 (Etherington & Taylor, 1967), with some slight streaking towards the anode. Acidification before electrophoresis gave the same pattern, which thus suggested that no acid-activated zymogen was present in the extracts, i.e. the extracts were free from pepsins 1 to 6 inclusive (Etherington & Taylor, 1967, 1969a) (Fig 2a). When the electrophoresis was performed at pH 5·0, three zones of activity were located. Two closely running zones of activity had migrated appreciably towards the anode with the third zone a short distance towards the cathode (Fig. 2b). An acidified tumour extract was then run in parallel with acidified fundic and pyloric mucosal extracts from non-tumourous stomachs that were adjusted to pH 5·0 before electrophoresis to destroy their peptic activity (Etherington & Taylor, 1970). The two zones which migrated towards the anode were found to migrate the same distance as the non-pepsin proteinase activity of the unininvaded fundic and pyloric mucosa (Fig. 2c). No cathodal-moving zone was evident for the unininvaded mucosal extracts.

Fig. 2. (a) Agar-gel electrophoresis at pH 5·0 of neutral (N) and acidified (A) extracts of a human gastric adenocarcinoma (patient 1). The positions of the different proteolytic zones (PZ) observed in normal gastric specimens are given for comparison. (b) Agar-gel electrophoresis at pH 8·2 of the same neutral extract. (c) Agar-gel electrophoresis at pH 8·2 of an acidified extract of a gastric adenocarcinoma (patient 4) with acidified extracts of unininvaded pyloric (F) and fundic (F) mucosa (patient 5).
Chromatography on DEAE-cellulose

The chromatogram obtained when a tumour extract was fractionated on DEAE-cellulose is shown in Fig. 3. The first gradient of 5-50 mM-potassium phosphate buffer, pH 8.0, eluted one proteinase and two further peaks were obtained in the 0-0.5 M-NaCl gradient. The pH-activity curves constructed on the 'cuts' of these three peaks are shown in Fig. 4. The activity in the first peak gave a nearly symmetrical curve with a sharp maximum at pH 3.6-3.7 and the curves obtained from the second and third peaks were identical with each other and asymmetrical with the maximum at pH 3.4.

Analysis of these three proteinases by agar-gel electrophoresis at pH 5.0 showed that each remained at the origin (Fig. 5). With electrophoresis at pH 8.2 the proteinase of peak I was just detectable towards the cathode and appeared to be unstable under these conditions. The proteinases of peaks II and III moved towards the anode with peak III showing the greater mobility. These two peaks thus represent the two anodal-moving zones of the whole-tumour extracts.

To refer more easily to the separate types of proteinase, we have given each a number. The proteinase with maximal activity at pH 3.6-3.9 and giving a symmetrical pH-activity curve has been called proteinase 1 and those proteinases giving asymmetrical pH-activity curves with the maximum at pH 3.3-3.4 and moving anodally at pH 8.2, have been called proteinases 2A and 2B respectively. The faster moving one is 2B.

Preliminary experiments with Amberlite CG 50 as used for the fractionation of the human pepsins (Etherington & Taylor, 1969a, 1970) indicated that these proteinases were bound to this resin more strongly than were the pepsins. Proteinase 2 was eluted as a single peak from the column before the elution of proteinase 1.
Effect of cysteine on the activity of proteinase 1

Cysteine has been shown to activate cathepsin D in several instances (Lapresle & Webb, 1960; Lundblad, Bernbäck & Widemann, 1966). However, with the proteinase 1 from the DEAE-cellulose column, cysteine was found to inhibit the digestion of denatured haemoglobin by 20% at 1.8 mM concentration and 26% at 3.6 mM concentration. The contrast between the proteinases of the neoplastic and normal tissues is thus marked.

Further purification of proteinase 1

Proteinase 1 (peak 1 in Fig. 3) was rechromatographed on CM-cellulose when two components were revealed (Fig. 6). The gradient of 10–50 mM-sodium acetate buffer, pH 5.5, eluted proteinase 1A but the major component proteinase 1B required much stronger conditions for desorption: 1.0 M-NaCl in 50 mM-potassium phosphate buffer, pH 7.5. Other minor peaks were also evident in this chromatogram. Attempts to obtain pure proteinase 2A and 2B, free of non-enzymic protein, have so far proved unsuccessful.

Digestion of the B-chain of oxidized insulin

The digestion with proteinase 1A was performed with 5 mg of the B-chain and 10 μg (crystalline swine pepsin equivalents) of the enzyme in a final volume of 4.3 ml. The pH readings were 3.5, 3.73 and 3.77 at 0, 6 and 24 h respectively. Proteinase 1B was allowed to digest 10 mg of

![Graph showing pH-activity curves for the digestion of denatured haemoglobin by enzyme fractions obtained from the DEAE-cellulose chromatogram of a human gastric adenocarcinoma extract (patient 1).]

Fig. 4. pH-activity curves for the digestion of denatured haemoglobin by the enzyme fractions obtained from the DEAE-cellulose chromatogram of a human gastric adenocarcinoma extract (patient 1). O, Chromatographic peak I; △, peak II; □, peak III.
FIG. 5. Chromatography of a human gastric adenocarcinoma extract on DEAE-cellulose (patient 1). (a) Analysis of the enzyme peaks by agar-gel electrophoresis at pH 5·0. (b) Analysis of the enzyme peaks by agar-gel electrophoresis at pH 8·2.

the B-chain and 21·5 μg (crystalline swine pepsin equivalents) of enzyme was used in a final volume of 4·2 ml. The pH readings were 3·5, 3·85 and 3·93 at 0, 4 and 24 h respectively.

The peptide maps prepared with a one-tenth sample of each digest revealed that most of the peptide spots were common to both digests, but differences existed mainly in the relative intensities of these spots. The cleavage sites in the B-chain of oxidized insulin determined for each proteinase are indicated in Table 1. Except for the splitting of the Ala(14)-Leu(15) bond by proteinase 1A the same bonds were cleaved by each proteinase but at different rates at the less susceptible sites.

DISCUSSION

The construction of pH–activity curves on whole-tumour extracts suggested the presence in a variable ratio of at least two types of proteinase. The use of ion-exchange chromatography together with agar-gel electrophoresis revealed in fact four different proteinases, denoted by the numerals 1A, 1B, 2A and 2B. Of the two main types of enzyme, the proteinases 1 displayed maximal activity at pH 3·7–3·9 and their pH–activity curves were nearly symmetrical. Proteinases 1A and 1B were only resolved by chromatography on CM-cellulose. They differed
from each other also by their extinction at 280 nm and to a minor extent by their action on the insulin B-chain.

The second type of enzyme, proteinases 2A and 2B, gave pH–activity curves that were broad and asymmetrical. Maximal activity occurred at pH 3.3–3.4 and declined slowly on the acid limb of the curve. The proteinases 2 were eluted after proteinase 1 from DEAE-cellulose, on which they were readily separated into the two forms, each with identical pH–activity curves.

Electrophoresis at pH 5.0 in agar gel did not resolve the different proteinases, which all remained at the origin. These proteinases were thus identified with the activity located at zone 7 in the pepsin zymograms prepared from gastric mucosal extracts (Etherington & Taylor, 1967, 1970). When the electrophoresis was conducted at pH 8.2, the proteinases 1 moved as a single zone towards the cathode and the proteinases 2 were separated into the two forms, A and B, migrating towards the anode. When chromatographed on Amberlite CG 50 (Etherington, 1967) the proteinases of type 2 behaved similarly to the non-pepsin proteinase fraction of normal gastric mucosa (Etherington & Taylor, 1970). Proteinases 1 have not been positively identified in normal gastric mucosal extracts, but proteinases similar to 1 and 2 have been recognized in extracts of intestinal mucosa (Etherington, 1967). The possibility of proteinases 1A and 1B in the tumour extracts arising from invaded muscle cells was investigated by agar gel electrophoresis at pH 8.2 of extracts prepared from uninvaded gastric muscle. A zone of activity indicating the presence of proteinases 1 was not found.

Electrophoretically and chromatographically the tumour proteinases thus resemble known
normal proteinases of the gut endoderm except for the uncertainty about the proteinases 1 in
gastric mucosa. This evidence would favour the view that the adenocarcinomatous cells had
failed to differentiate in such a way as to elaborate only the normal endodermal proteinases.
When comparing the tumour proteinases with the proteinases of non-gut tissues, the chromato-

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← - - minor, ← moderate, ← major sites of cleavage.

graphic and electrophoretic properties of proteinase 1 together with its pH–activity curve
showed some similarities to rabbit spleen cathepsin D (Lapresle & Webb, 1960, 1962), human
thyroid proteinase (Lundblad et al., 1966) and bovine spleen cathepsin D (Press, Porter &
Cebra, 1960), but the tumour proteinases 1 differed in being inhibited rather than activated
by cysteine. This evidence suggests that a failure to elaborate pepsins with retention of the
Proteinases of gastric carcinomata

endodermal proteinases is too simple an explanation. The electrophoretic and chromatographic behaviour of the proteinases 2 is similar to that of cathepsin E of rabbit bone marrow (Lapresle & Webb, 1962). It might therefore be reasonable to regard the tumour proteinases 2 and the zone 7 gastric proteinases as resembling the normal cathepsins of non-gut tissues, and this evidence too would support a simple failure of differentiation.

The two proteinase 1 enzymes from the gastric tumour displayed similar modes of action when digesting the B-chain of oxidized insulin. Differences were limited to the moderate splitting by proteinase 1A of the Ala(14)-Leu(15) bond and the relative rate at which the bonds at Phe(1)-Val(2), Glu(13)-Ala(14) and Gly(23)-Phe(24) were cleaved. The specificities were wider than those for bovine spleen cathepsin D (Press et al., 1960) and substantially different from that for rabbit spleen cathepsin D (Lapresle & Rangel, 1966). The tumour proteinase 1 do resemble, however, a bovine lung cathepsin (Dannenberg & Smith, 1955) except that Leu(11)-Val(12) bond was weakly split by the latter. A proteinase from hog thyroid (Kress, Peanasky & Klitgaard, 1966) which is probably analogous to the human thyroid proteinase, split all but one of the bonds susceptible to the tumour proteinases. The specificities found for a number of cathepsin D preparations have shown certain differences of action (Bohley, Kirschke, Langner, Ansorge, Wiederlanders & Hanson, 1971), which for bovine tissues at least indicate that cathepsin D may be organ specific. The neoplastic proteinases 1 thus attack the insulin B-chain somewhat more comprehensively than do many of the cathepsins D. When compared, on the other hand, with the normal human pepsins, the tumour proteinases 1A and 1B cleave only those bonds that are susceptible also to the pepsins. Overall they resemble the human pepsin 5 as closely as they resemble the cathepsins of bovine lung and hog thyroid. The evidence from their action on the B-chain would thus on balance favour the hypothesis that the proteinases 1 are modified pepsins.

Taking all the evidence together it is clear that no firm choice between the two hypotheses can be made. Whether the tumour proteins are normal cathepsins or whether they represent an intermediate enzyme form can only be decided from protein structural studies, and from the elucidation of the sites of action on the insulin B-chain of proteinases from tumours other than the stomach and of normal proteinases 1A and 1B, if such enzymes exist. But it is clear that no enzymes exactly like the proteinases 1 of gastric adenocarcinomata have yet been found in normal tissues.

ACKNOWLEDGMENTS

Thanks are due to the surgeons of the Liverpool Royal Infirmary for the provision of surgical material and to Miss P. Gale for skilled technical assistance.

REFERENCES


