

# The pepsinogens of human gastric mucosa

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**SUMMARY** Fundic mucosal pepsinogens reveal six major bands of protease activity on gel electrophoresis and four minor bands. Antral mucosa shows two major bands of activity; the four rapidly moving bands found in fundic mucosa are found in lesser activity in the antral mucosa. Duodenal mucosa shows only one slowly moving band of protease activity. There is a significant difference in the pepsinogen pattern when the uninvolved fundic mucosa of gastric ulcer patients is compared with that of cancer patients.

A satisfactory method is described for separating the pepsinogens on column chromatography.

The pepsinogens of human gastric mucosa have been the subject of several studies using electrophoretic and chromatographic techniques (Uriel, 1960; Seijffers, Segal, and Miller, 1963; Tang and Tang, 1963; Kushner, Rapp, and Burtin, 1964; Rapp, Aronson, Burtin, and Grabar, 1964; Hanley, Boyer, and Naughton, 1966; Taylor, 1968; Samloff, 1969); it has been shown that gel electrophoresis is superior to other fractionation procedures (Taylor, 1968) but the findings have been divergent. The present study re-investigates this topic using the collective experience of other workers (Uriel, 1960; Seijffers *et al*, 1963; Tang and Tang, 1963; Kushner *et al*, 1964; Rapp *et al*, 1964; Hanley *et al*, 1966; Taylor, 1968; Samloff, 1969), defines the pepsinogen pattern of human gastric mucosa, explains the divergent findings of earlier investigators, investigates the role of gastric disease in the determination of the pepsinogen pattern, describes a satisfactory column chromatographic method of pepsinogen fractionation, and relates the mucosal pepsinogen pattern to the pepsin patterns of human gastric juice.

## Methods

### TISSUE PROCUREMENT AND PREPARATION

Human gastric mucosa was obtained from surgically resected specimens and stored at  $-15^{\circ}\text{C}$  before use. After removal from the muscle, the mucosa was homogenized in ice-cold 0.1 M phosphate buffer pH 7.3 and centrifuged at 17 300 *g* at  $4^{\circ}\text{C}$  for 20 minutes. The supernatant was used as the source of pepsinogens without further purification.

Sixteen samples of mucosa uninvolved in the gastric lesion from eight patients were taken from sites adjacent to areas of mucosa which were identified as fundic, antral, or duodenal mucosa using histological techniques.

The specimens were from patients with duodenal ulcer (3), gastric ulcer (14), and gastric carcinoma (15). In none included in this study was there any doubt regarding the diagnosis.

To determine the effect of the presence of pepsin in the mucosal homogenates on the electrophoretic pattern of the pepsinogens, two fundic mucosal homogenate samples were acidified to pH 2-3 for 10 to 40 seconds and then titrated to pH 5-6. These samples were assayed for pepsin in the presence of pepsinogen using the milk-clotting method of Seijffers *et al* (1963) as well as being electrophoresed.

### ELECTROPHORESIS OF MUCOSAL HOMOGENATES

The method was that used by Samloff (1969) with minor modifications. A solution of 1.5% agar (Difco Laboratories, Michigan) in 0.05 M barbital (BDH) buffer, pH 8.3, was poured on a 20 × 40 cm glass plate to form a layer about 1-2 mm thick. The same buffer was placed in the electrophoresis tanks, which were connected to the gel by wicks of four thicknesses of Whatman no. 3 paper. The samples, after mixing with warm 2% agarose (Calbiochem) at 50 to 60°C, were pipetted into slits 1 × 0.1 cm cut 5 cm from the cathode end of the gel. This technique was carried out so rapidly that variations in the temperature of the agarose were not observed to alter the electrophoretic pattern. Differing amounts of agarose were used to

give different concentrations of pepsinogen, so that a suitable amount of activity was available for the detection of individual proteolytic zones. The entire gel was covered with a sheet of thin plastic wrap to prevent evaporation during electrophoresis.

Electrophoresis was carried out for four hours at 40 v per cm, using a Savant high voltage electrophoresis unit with a flat plate. The gel was kept between 10 and 15° by resting it on a cooled metal plate.<sup>1</sup>

The proteolytic bands were detected by the method of Uriel (1960) with slight modifications. The gels were soaked in 0.6% bovine haemoglobin (Sigma Laboratories) which was 0.06 M with respect to HCl (about pH 1.8) for 10 minutes. After pouring off this solution, the gels were incubated at 37°C in a humid atmosphere for an hour, fixed in 5% acetic acid in 50% ethanol for at least 10 minutes, stained with 5% amido black for 30 minutes and destained overnight with 5% acetic acid.

#### CHROMATOGRAPHIC ANALYSIS OF PEPSINOGENS AND SUBSEQUENT GEL ELECTROPHORESIS

Whatman DEAE cellulose (grade DE32) was pretreated with acid and alkali as recommended by the manufacturers and equilibrated with 0.02 M phosphate buffer pH 5.2. This was used to make a column 70 × 1.2 cm, to which 1.2 mg pepsinogen in 2.4 ml of the homogenate supernatant was applied. After washing with 150 ml of buffer, a linear sodium chloride gradient was achieved by connecting the column to cylinders of buffer which were 0.1 M and 0.4 M with respect to sodium chloride. Fractions of 5 to 6 ml were collected using an automatic fraction collector. The temperature remained between 15 and 20°C during elution. Chloride concentration in the effluent was determined using Sigma Laboratories chloride test kits.

Proteolytic activity was determined by a modification of the method of Anson and Mirsky (1932): 0.5 ml column effluent + 2.0 ml 0.4 M glycine buffer pH 2.2 was incubated with 0.4 ml 4% bovine haemoglobin at 37°C for 15 minutes. The reaction was terminated by the addition of 1 ml 20% TCA, the mixture centrifuged, and 1 ml of the supernatant assayed for tryosine-like material using Folin-Ciocalteu reagent.

Several fractions from each peak were pooled, dialysed for about 20 minutes against distilled water, and concentrated by evaporation in front of a fan. These samples were divided into two: one half was electrophoresed as before, and the other half was acidified and electrophoresed using the same tech-

nique as for pepsinogens except for the replacement of the barbital buffer by 0.02 M citrate-phosphate buffer, pH 4.8. In this case, human gastric juice was used as human pepsin standards.

#### pH ACTIVITY CURVES USING HAEMOGLOBIN SUBSTRATE

Several fractions from pepsinogen peaks 2 and 4 were pooled, acidified, and the pH activity curves of these pepsins were obtained using slight modifications of the Anson and Mirsky (1932) method. Glycine buffer 0.4 M was used for pH 1.2-2.4, and citrate-phosphate buffer 0.4 M was used for pH 2.2-3.5. Bovine haemoglobin was titrated to each pH using HCl and NaOH, and its final concentration in the reaction mixture was 6.5 mg/ml.

#### INFLUENCE OF ELECTROPHORETIC TECHNIQUE ON PEPSIN ELECTROPHORETIC PATTERN

Samples of fundic, antral, and duodenal mucosal homogenates were applied to a gel prepared as before but a voltage of 10 volts per cm was applied for eight hours instead of the usual high voltage of 40 v/cm.

#### Results

##### GEL ELECTROPHORETIC PATTERNS AND SITE OF ORIGIN OF TISSUE

The nomenclature used will be based on the electrophoretic mobility and the size and constancy of each band; consequently we shall describe major bands which were always obvious on gel electrophoresis and were clearly defined on column chromatography and minor bands which were less obvious on gel electrophoresis, often diffuse or bifid and were not obvious on column chromatographic analysis.

##### MAJOR BANDS

There were six major bands of protease activity. All were present in fundic mucosa, only the two slowly moving bands were prominent in antral mucosa and only one, the most slowly moving of the six present in fundic mucosa, was present in duodenal mucosa (fig 1).

The difference between fundic and antral mucosa represents merely a difference in the ratio of the activity of bands 1-4 to 5-6. If the time of incubation of antral mucosa was increased, faint bands 1-4 appeared; this is obvious in figure 1. The histological tissue type and the electrophoretic pattern type were found to agree in six out of nine samples with a fundic electrophoretic pattern, three out of five with an antral electrophoretic pattern, and in both samples with a duodenal electrophoretic pattern. The

<sup>1</sup>Details will be supplied by the authors on request.

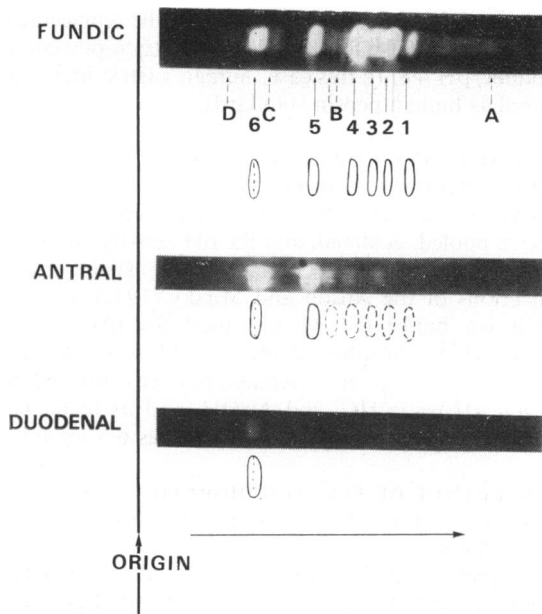


Fig 1 Electrophoretic pattern of pepsinogen separation in agar gel comparing fundic, antral, and duodenal mucosa.

variations between histological identification and pepsinogen pattern may have been due to the tissue examined histologically not representing the tissue chosen for electrophoresis.

#### MINOR BANDS

**A**  
This is a diffuse area with greater electrophoretic mobility than the main pattern. It is not pepsin, as human pepsins were found to be inactivated during electrophoresis under the conditions used here. Also the area tends to decrease in activity as time of exposure to acid conditions increases, whereas the milk-clotting assays showed clear increases in the amount of active pepsin as the time of exposure to acid was increased.

#### **B, C, and D**

**B** consists of one or sometimes two discrete faint bands between major bands 4 and 5. **C** is a discrete faint band preceding band 6, and area **D** is less discrete following band 6. As time at pH 2-3 increases from 0 to 40 seconds, bands 5 and 6 become weaker while minor bands **B** and **C** increase in strength.

#### INFLUENCE OF GASTRIC DISEASE ON PEPSINOGEN PATTERNS

The number of major bands observed did not vary

with disease. However, when the order of magnitude of the bands 1, 2, 3, and 4 was considered, a significant difference between the gastric ulcer and the gastric carcinoma group did appear (table). Of these four bands, band 2 was the largest band more commonly in gastric ulcer than in gastric carcinoma patients ( $P < 0.01$ ) and band 4 was more commonly the largest band in the carcinoma than the ulcer group ( $P \approx 0.01$ ). The incidence of minor bands **B** and **C** was similar in both groups ( $P = 0.1$ ). Bands 5 and 6 showed no change associated with disease.

Diagnosis	No. Showing Largest Band as:				Total
	1	2	3	4	
Gastric cancer	1	2	2	10	15
Gastric ulcer	1	9	1	3	14

Table Band size in gastric ulcer and gastric cancer groups.

#### INFLUENCE OF OTHER PATIENT FACTORS ON PEPSINOGEN PATTERNS

The influence of age, sex, and smoking habits was investigated for 25 patients in this survey. No significant difference was found either in the number of minor bands detected nor in the particular band showing the greatest activity.

#### CHROMATOGRAPHY AND SUBSEQUENT GEL ELECTROPHORESIS OF PEPSINOGENS

On column chromatography of fundic mucosal homogenates six well defined peaks were obvious (figs 2 and 3). Subsequent electrophoresis of the concentrated fractions from each peak showed that each was formed by an electrophoretically distinct pepsinogen with slight contamination by the adjacent pepsinogens due to overlapping of the peaks. The pepsinogens which are slower on electrophoresis are eluted from the column before those which are faster on electrophoresis, ie, the electrophoretic mobility of the pepsinogens is directly proportional to the molarity of NaCl required to elute the pepsinogen from the column.

In one case, proteolytic activity was assayed before and after column chromatography, and the recovery was found to be 65%. However the sizes of the peaks are quite comparable to the sizes of the bands on electrophoresis. This is most noticeable when comparing the chromatographic analysis of pepsinogens from ulcer and cancer patients' tissue, where the largest peak is formed by pepsinogens 2 and 4 respectively (figs 2 and 3).

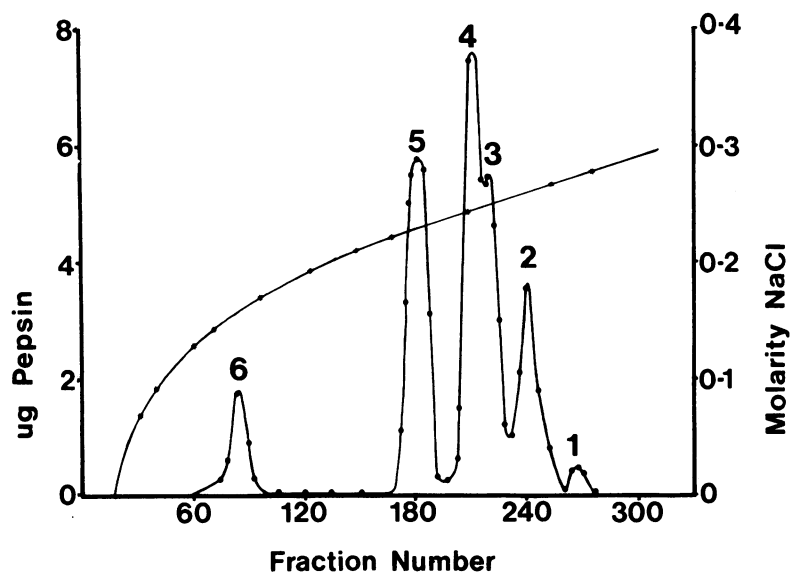


Fig 2 Elution pattern from DEAE cellulose column of pepsinogens from mucosal homogenate of a gastric cancer patient.

**INFLUENCE OF ELECTROPHORETIC TECHNIQUE ON PEPSINOGEN ELECTROPHORETIC PATTERN**

The lower voltage was sufficient to separate bands 5 and 6 from each other and from bands 1-4. However, bands 1-4 separated into two bands rather than four. Thus the low voltage method is adequate for antral or duodenal pepsinogens, but does not give complete separation of all six fundic pepsinogens.

**CONVERSION OF PEPSINOGENS TO PEPSINS**

Each of the pepsinogens 1-4 gave rise to a single pepsin band on electrophoresis; pepsinogen 5 gave rise to two pepsin bands of equal activity. The pepsins derived from pepsinogens 2 and 4 had identical electrophoretic properties. Pepsinogen 6 was insufficiently concentrated for any activity to be detected. These findings are represented schematically in figure 4.

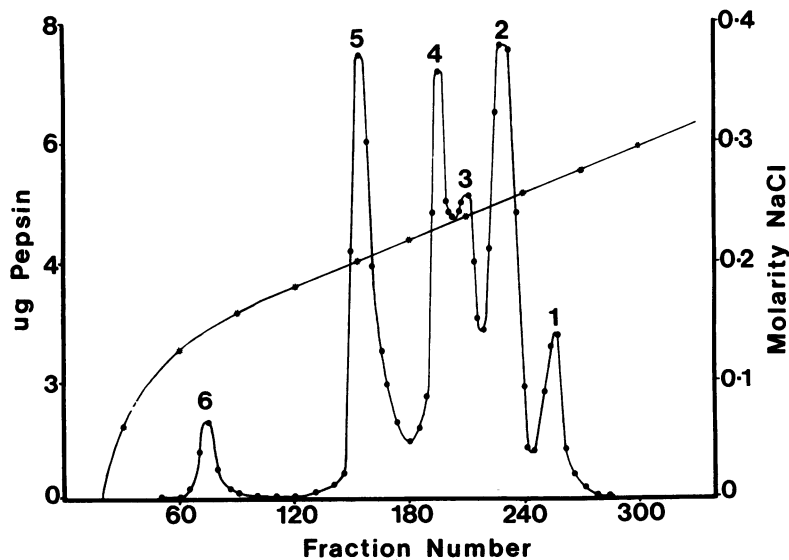


Fig 3 Elution pattern from DEAE cellulose column of pepsinogens from mucosal homogenate of a gastric ulcer patient.

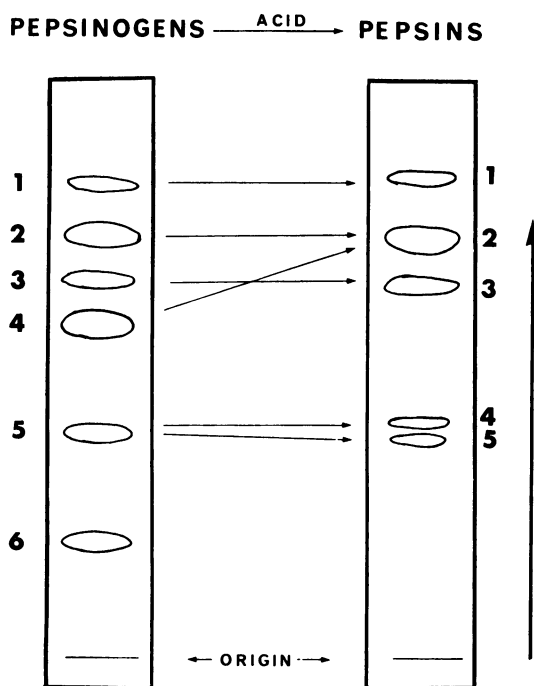


Fig 4 Relationship of mucosal pepsinogens to gastric juice pepsins

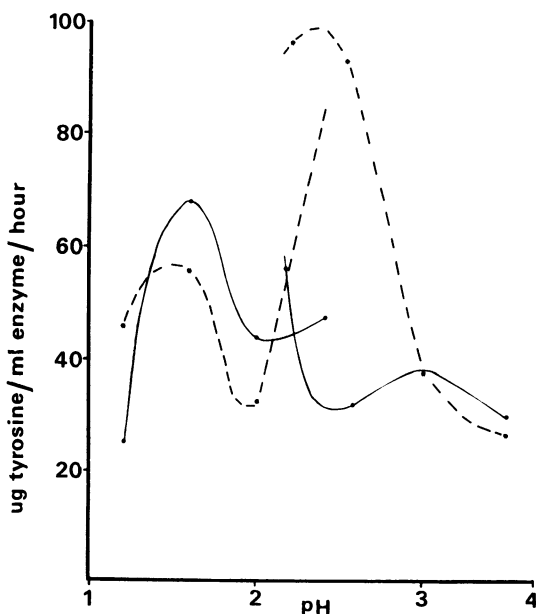


Fig 5 pH activity curves of the pepsins from pepsinogens 2 (---) and 4 (—) with bovine haemoglobin as substrate.

#### pH ACTIVITY CURVES

Both pepsins from pepsinogens 2 and 4 gave rise to activity curves of similar overall shape in the region pH 1.2-2.0. However, in the region pH 2.2-3.5 the activity curves showed different pH optima, and although both pepsins had similar activity in the pH 1.2-2.0 region, the pepsin from pepsinogen 2 was markedly more active in the pH 2.2-2.8 region (fig 5).

#### Discussion

A great stimulus to the study of pepsinogens was provided by the introduction of gel electrophoresis and the use of sensitive detection methods; these are well demonstrated in the sophisticated studies of Samloff (1969). A divergence in the number of bands characterized the reported work. The present study supported the concept suspected by others that this variation was due to the techniques used. Poor resolution of the bands and consequently the finding of a smaller number of bands resulted from diffusion of areas of protease activity. This can be reduced by shortening the time of the whole procedure by high voltage electrophoresis as suggested by Samloff (1969); the use of the sensitive detection methods derived by Uriel (1960) contributes further to the value of the technique.

Using the gel electrophoresis techniques outlined here, it is possible to separate the pepsinogens of human gastric mucosa into a maximum of six major and four minor areas of activity. These may all be found in fundic mucosa, whereas antral mucosa shows only two major areas of activity, and duodenal mucosa shows only one area of activity. We do not find, as Samloff (1969) suggested, that a qualitative difference exists between fundic and antral mucosa. Bands 1-4 are present in antral mucosa though proportionally small when compared with bands 5-6. His failure to detect these pepsinogens by immunofluorescent techniques in antral mucosa could merely reflect their low activity there relevant to fundic mucosa; it is noted that he did observe isolated islets of 'fundic' pepsinogens in the antral region, as is demonstrated in fig 6 of his article (1971).

When the patterns obtained from mucosa from patients with gastric ulcer and gastric carcinoma were compared only the order of magnitude of the major bands was found to differ. In gastric ulcer patients, band 2 predominated and in gastric cancer patients band 4 predominated. It is difficult to know which group is abnormal but as band 4 predominated in the mucosa of the three patients with duodenal ulcer, we feel the large band 2 pepsinogen found

in the mucosa of gastric ulcer patients may reflect an abnormality associated with gastric ulcer.

Since very brief acidification caused an increase in minor areas B and C, it is possible that these areas are formed by the action of acid on the pepsinogens in the mucosa before homogenization in buffer and are not pepsinogens present in the living cell.

An interesting finding of the present study not previously accomplished was the separation of the electrophoretically distinct pepsinogens on gel electrophoresis by column chromatography. Using a similar technique, Seijffers *et al* (1963) only obtained three well defined fractions. This difference in findings could be attributed to the change in the qualities of DEAE cellulose over the last 10 years and to the shorter column used by these workers. Also Seijffers *et al* (1963) applied smaller amounts to homogenate with the result that peaks 3 and 1 would not have been distinguishable from peaks 4 and 2 respectively; peak 6 of the present study is small, as is seen in figs 2 and 3, and is possibly the same as the very small peak sometimes present at the beginning of some of the chromatograms of Seijffers *et al* (1963).

This study has shown that two pepsinogens (2 and 4) gave rise to pepsins with identical electrophoretic mobility on acid activation and pepsinogen 5 gave rise to two pepsins. The pepsins obtained after acidification of the pepsinogens need further characterization using different substrates. It is of interest to note the relatively great activity at a pH frequently found in gastric juice of the pepsin derived from pepsinogen 2. This was the pepsinogen that was usually the major band in gastric ulcer patients when the latter group was compared with non-ulcer patients.

As this study has emphasized, the study of pepsinogens has been bedevilled by variations in findings resulting from the different techniques used. Also no satisfactory method of terminology has been developed. Samloff (1969) labelled each band numerically in relation to anodal migration properties. This classification has the deficiency that it does not take into consideration the size and degree of definition of each band and the separation found on

column chromatography. Our band A represents band 1 of Samloff, our bands 1-4 bands 2-5 of Samloff, band B corresponds with band 6, band 5 with band 7 and bands 6, C and D with Samloff's SMP. We think that it is not appropriate to consider band A a single protease because of its diffuse nature. We agree with both Kushner *et al* (1964) and Samloff (1969) that bands C, 6, and D may not be pepsinogens because they are resistant to acidification and later neutralization; however, the pH activity curve of this protease using haemoglobin as a substrate shows a maximum at low pH levels similar to the curves produced by bands 1-5.

This work is supported by the National Health and Medical Research Council of Australia, the Australian Tobacco Research Foundation, and anonymous donors.

The helpful advice of Dr M. A. Messer, of the Department of Biochemistry, University of Sydney, is gratefully acknowledged.

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