The Pepsins of Normal Human Gastric Juice

By D. J. ETHERINGTON* AND W. H. TAYLOR

Department of Chemical Pathology, United Liverpool Hospitals, Liverpool 3

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1. The frequency of occurrence, under defined conditions, of the different human pepsins in the gastric juices of 50 normal subjects was investigated by agar-gel electrophoresis. 2. From a total of eight proteolytic zones located in the zymograms, no significant differences of occurrence existed between the sexes, or between subjects with or without gastric symptoms. 3. Two zones, numbered 3 and 5, occurred in all normal gastric juices. Zone 3 always exhibited the greatest proteolytic activity, then zone 5. The remaining enzymic zones were less well-marked and occurred less frequently. 4. A minor zone, 3a, was demonstrated within zone 3. The corresponding pepsin, 3a, has a mobility towards the anode 6–7% greater than has pepsin 3. 5. Of the eight zones, 1, 2, 3, 3a and 5, at least, represent unique pepsins.

The human stomach synthesizes and secretes into gastric juice more than one proteolytic enzyme with optimum activity at about pH 2.0 (Taylor, 1956, 1959a). Richmond, Tang, Wolf, Trucco & Caputto (1958) independently separated a minor proteinase, which they named 'gastricsin', from human gastric juice by ion-exchange chromatography. This enzyme was subsequently shown to differ from pepsin by its biological activity (Tang, Wolf, Caputto & Trucco, 1959) and by its molecular weight and amino acid composition (Mills & Tang, 1967).

Gastric mucosal extracts from normal subjects similarly were shown to contain more than one pepsin (Taylor, 1956, 1959b), one of which was named 'pyloric pepsin'. Seijffers, Segal & Miller (1963a,b) subsequently found three pepsinogens by chromatography of gastric mucosal extracts on DEAE-cellulose. These on activation yielded four pepsins. By a similar technique, Kushner, Rapp & Burtin (1964) claimed to have found a total of four pepsinogens, which yielded five electrophoretically distinguishable pepsins.

The technique of electrophoresis in agar gel was successfully used by Kushner *et al.* (1964) for the demonstration of proteolytic zones caused by enzymes in gastric mucosal extracts and in chromatographic fractions, and was subsequently applied to the investigation of the pepsins of gastric juice (Hirsch-Marie, Burtin & Conte, 1965). This technique provides a ready means of identifying the pepsins that are present in the fractions prepared by different workers. We have suggested a nomenclature for the pepsins, based on this technique, in

an attempt to resolve the confusion that has arisen in recent years (Etherington & Taylor, 1967). A total of eight zones possessing proteolytic activity can be demonstrated by agar-gel electrophoresis, and these have been numbered in order of decreasing mobility. In earlier work only seven zones were distinguished, and so numbered. Subsequently zone 3 was found to contain a further zone. In order to prevent a further confusion of nomenclature, this zone has been named 3a.

The results presented in this paper establish the frequency of occurrence of each of the zones in normal human subjects and determine which of the zones can be assigned to unique pepsins.

MATERIALS AND METHODS

Gastric juice. Specimens were collected by pernasal intra-gastric tube from seven normal volunteers, in one of whom histamine (2mg.) was the stimulant and in the others pentagastrin (0.6 µg./kg. body wt.), and from hospital patients undergoing augmented histamine tests (Kay, 1953). These patients were subsequently shown to be free from gastric disease by radiological investigations and sometimes also by gastroscopy, by gastric camera and by laparotomy. They were also free from diseases such as carcinomatosis and anaemia, which are known to diminish gastric secretion. They were divided into two groups: (i) 21 subjects with no gastric symptoms, and (ii) 22 subjects with some gastric symptoms. The total number of gastric juices examined was thus 50. Samples of juice were adjusted to pH 4.0 with 1M-NaOH before storage at –15°.

Agar-gel electrophoresis. The method of Uriel (1964) was used with slight modifications. Glass plates (20 cm. × 10 cm.) were coated with a 1% (w/v) solution of agar (Ionagar no. 2; Oxoid Ltd., London, E.C.4) in 25 mM-sodium acetate buffer, pH 5.0. Gastric-juice samples, suitably diluted with 1 mM-HCl (usually tenfold), were mixed with equal volumes of

* Present address: Agricultural Research Council Meat Research Institute, Langford, Bristol BS18 7DY.

double-strength agar at 50° and pipetted into 8 mm. × 1 mm. slots cut into the agar, 5 cm. from the cathode end. The samples were kept at 50° for only 2–3 min. and no detectable loss of activity, or alteration of detectable zones, was induced. Hanley, Boyer & Naughton (1966) found no loss of activity when pepsinogens were kept at 46° for 30 min. Crystalline swine pepsin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) (0.5 mg./100 ml. in 1 mM-HCl) was used as a standard marker. Electrophoresis was conducted in a cold-room at 4° for 3 hr., with a potential gradient of 8 v/cm. The electrophoresis tank contained 0.1 M-sodium acetate buffer and wicks were prepared from four thicknesses of Whatman 3MM paper. The gel surfaces were covered with a sheet of polythene (0.01 in. thick) to prevent evaporation during electrophoresis.

Zones of proteolytic activity were detected by the method of Uriel (1960). Agar plates were immersed for 1 hr. in a solution of 0.2% (w/v) human globin (prepared by the method of Ito, Guroff & Udenfriend, 1964) in 0.2 M-glycine-HCl buffer, pH 1.9. This solution was then decanted and the agar plates, in a covered tray, were incubated at 37° for 2 hr. The agar was fixed and stained with Ponceau S (Uriel, 1964).

Ion-exchange chromatography. Amberlite CG-50 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) was regenerated by the method of Hirs, Moore & Stein (1953) and packed into columns (20 cm. × 2.5 cm.). Gastric-juice samples (30–100 ml.) were concentrated with Carbowax to approx. 10 ml. and then dialysed against the 0.2 M-sodium citrate buffer, pH 2.9, that was used to equilibrate the columns. After being loaded, the columns were developed by the procedure of Richmond, Caputto & Wolf (1957) with 0.2 M-sodium citrate buffers, but with a proportionately linear gradient established between buffers of pH 2.9 and 6.0. The concentration of Na⁺ was made up to 0.36 M by the addition of NaCl. A volume of 1350 ml. of buffer was used to develop each column at a flow rate of 2–3 ml./min.

DEAE-cellulose (Whatman, grade DE52) was prepared and packed according to the manufacturers' instructions. A 10 ml. sample of gastric juice was dialysed against 0.1 M-sodium acetate buffer, pH 5.3, and applied to a column (22 cm. × 1 cm.) equilibrated with the same buffer. The column was developed by a system similar to that used by Seijffers *et al.* (1963a). After the column had been washed free of unadsorbed protein, a convex concentration gradient was established with NaCl in the 0.1 M buffer. A beaker was used as the mixing vessel, containing 0.17 M-NaCl in 270 ml. of buffer. A conical flask was used as the reservoir and this contained 0.21 M-NaCl in 280 ml. of buffer. After the passage of a further 50 ml. of this latter buffer, the column was developed with a 250 ml. linear gradient to 1.0 M-NaCl. A flow rate of 0.5–1.0 ml./min. was maintained.

Analysis of column effluents. Effluent fractions were analysed for protein by measuring E_{280} with an Optica (Gateshead) u.v. spectrophotometer. The pH was determined with a Vibron 39 A pH-meter (Electronic Instruments Ltd., Richmond, Surrey) and chloride was determined by coulometric titration with an EEL chloride meter (Evans Electrochemical Ltd., Harlow, Essex). Proteolytic activity was determined by the method of Anson & Mirsky (1932) as modified by Hanley *et al.* (1966), but with 0.1 ml. of eluate in a 2 ml. digestion mixture buffered at pH 1.9. Tubes were incubated at 37° for 30 min. or 1 hr., depending on the concentration of pepsin in the eluate.

RESULTS

Agar-gel electrophoresis at pH 5.0 revealed a maximum of eight zones of proteolytic activity, as reported by Etherington & Taylor (1967). Typical zymograms of normal gastric juice are shown in Fig. 1, together with a diagram indicating the location of zones 1–7. Table 1 records the frequency with which these zones were present in the different samples of gastric juice. Only the pepsin zones 3 and 5 were present in every juice.

The normal volunteers and the two groups of patients showed no significant differences in the frequency with which zones 1, 2, 4, 6 and 7 occurred. Zone 2 seemed to occur more frequently (10 patients, 45.5%) in the patients with symptoms than in those without symptoms (4 patients, 19.0%), but the difference is not significant ($0.1 > P > 0.05$). There were no significant differences between the sexes in the frequency of occurrence of the seven zones.

Effect of storage. Zone 4 was found to weaken in intensity with storage of the gastric juice. As Seijffers, Miller & Segal (1964a) have demonstrated that human pepsin-pepsin inhibitor complexes are stable above pH 5.0, but dissociate with digestion of the inhibitor near pH 4.0 (the pH of storage), the possibility of zone 4 (and of any other zone) representing such a complex was investigated. One portion of fresh gastric juice was titrated to pH 3.5 with 0.1 M-sodium hydroxide and incubated at 37° for 20 min. before being subjected to agar-gel electrophoresis. Herriott (1941) found that the

Fig. 1. Agar-gel electrophoresis at pH 5.0 of samples of human gastric juice (1–4), illustrating the locations of the eight zones of proteolytic activity, which are shown diagrammatically in (5). Crystalline swine pepsin (ST) was run as a standard marker. O, Origin; +, anode.

Table 1. Frequency of occurrence of the different zones of proteolytic activity of normal human gastric juice after electrophoresis on agar gel at pH 5.0, with human globin as substrate and incubation at pH 1.9 for 2 hr.

Subjects	No. of subjects	No. showing proteolytic zones						
		Zone 7	6	5	4	3	2	1
Normal male volunteers	7	0	0	7 (100%)	2 (28.6%)	7 (100%)	2 (28.6%)	3 (42.9%)
Without gastric disease and symptoms								
Males	10	2	1	10	5	10	2	2
Females	11	3	0	11	7	11	2	7
All	21	5 (23.8%)	1 (4.8%)	21 (100%)	12 (57.2%)	21 (100%)	4 (19.0%)	9 (42.9%)
Without gastric disease but showing some symptoms								
Males	15	4	1	15	10	15	7	10
Females	7	1	0	7	3	7	3	2
All	22	5 (22.7%)	1 (4.5%)	22 (100%)	13 (59.1%)	22 (100%)	10 (45.5%)	12 (54.6%)
Total								
Males	32	6 (18.8%)	2 (6.3%)	32 (100%)	17 (53.1%)	32 (100%)	11 (34.2%)	15 (46.9%)
Females	18	4 (22.2%)	0 (0%)	18 (100%)	10 (55.6%)	18 (100%)	5 (27.8%)	9 (50%)
All	50	10 (20%)	2 (4%)	50 (100%)	27 (54%)	50 (100%)	16 (32%)	24 (48%)

Fig. 2. Agar-gel electrophoresis at pH 5.0 of two gastric juices, which had been either (P) preincubated at pH 3.5 and 37° for 20 min., or (A) adjusted directly to pH 5.0. ST is the standard marker, crystalline swine pepsin. Zone 4 disappeared after incubation.

Fig. 3. Agar-gel electrophoresis at pH 5.0 of two gastric juices: the activity was located after incubation at 37° for (a) 2 hr. and (b) 16 hr. ST is the standard marker of crystalline swine pepsin.

optimum pH for digestion of swine pepsin inhibitor was 3.5. A second portion of gastric juice was titrated directly to pH 5.0 and not incubated at 37°. Fig. 2 shows that zone 4 disappeared after pre-incubation at pH 3.5, but was present in each control sample. Activity at the other zones was unaltered.

The fate of the substance(s) that disappear is not known. Achlorhydric gastric juices. Achlorhydric or hypochlorhydric gastric juices from patients with and without gastric disease frequently exhibited activity at zone 6, whereas those juices containing a normal concentration of hydrochloric acid rarely exhibited

Fig. 4. Chromatography of gastric juice on a 20 cm. $\times$ 2.5 cm. column of Amberlite CG-50. Pepsin activity was eluted by a proportionately linear gradient between 0.2M-sodium citrate buffers of pH 2.9 and 6.0. The concentration of Na^+ was maintained at 0.36M by the addition of NaCl. Fractions (8 ml.) were collected. —, E_{280} (total protein); $\circ$, E_{700} (peptic activity); $\square$, pH.

Fig. 5. Agar-gel electrophoresis at pH 5.0 of the two pepsin peaks (I and II) eluted from the column of Amberlite CG-50. ST is the standard marker of crystalline swine pepsin.

this activity (Table 1). Incubation of such gastric juice samples at 37° and pH 2.0 for 10 min., before electrophoresis in agar gel at pH 5.0, caused the activity at zone 6 to disappear, which suggests that this zone represents a remaining zymogen.

Effect of increasing the time of digestion. Zones 1 and 2 were often only faintly visible and usually somewhat streaked. Extended incubation of the agar plate after electrophoresis, for 16 hr. at 37° for two samples of gastric juice, in which neither zone was detected after 2 hr. at 37°, revealed the presence of a zone 7 and of an apparent zone 2 for one gastric juice and of a zone 1 for the second gastric juice (Fig. 3). The remaining zones showed confluency because of overdigestion.

Components of zone 3. The major pepsin zone, 3, often showed a slight elongation at the leading edge, which suggested that a second, slightly faster, component, 3a, was present (see Fig. 1). During preparative electrophoresis in agar gel at pH 5.0

(Etherington, 1967), pepsin 3a was obtained almost free of pepsin 3, and showed a mobility towards the anode 6–7% more than that of pepsin 3. The frequency of occurrence of pepsin 3a in each gastric juice is not known, as it is easily masked by pepsin 3.

Zones 5 and 7. Pepsin 5 was present in each gastric juice examined and was always present in a smaller amount than pepsin 3. The activity at zone 7 was weakly demonstrated in ten gastric juices, but was more readily detected after 16 hr. incubation of the agar plate after electrophoresis (Fig. 3).

Ion-exchange chromatography. Chromatography of gastric juice on Amberlite CG-50 gave two partially resolved peaks of activity (Fig. 4) as found by Richmond *et al.* (1957). Rechromatography with a shallower pH gradient failed to improve the separation. Analysis of these pepsin peaks by agar-gel electrophoresis indicated that peak I contained mainly pepsin 3 and peak II contained pepsin 5 with some pepsin 3 (Fig. 5). Pepsin 2 was eluted with pepsin 3 in peak I. Pepsin 1 was not certainly identified in these analyses.

Gastric juice was chromatographed on DEAE-cellulose (Fig. 6) when four partially resolved pepsin peaks were eluted with the sodium chloride gradient used by Seijffers *et al.* (1963a). A fifth pepsin peak was eluted when the influent concentration of sodium chloride was raised to 1.0M. Analysis of these peaks by agar-gel electrophoresis at pH 5.0 (Fig. 7) showed that the first peak, which emerged at the start of the gradient, gave activity at zones 3 and 4. Peak II contained pepsin 5 that had been eluted with a pepsin 3. Peaks III and IV each contained a pepsin 3 also, but, as the mobilities were almost identical, it was not possible to assign pepsin 3a to any one of these three peaks. Pepsin 1 was eluted in peak V, the pepsin not found by Seijffers

Fig. 6. Chromatography of gastric juice on a 22 cm. $\times$ 1 cm. column of DEAE-cellulose equilibrated with 0.1 M sodium acetate buffer, pH 5.3. Pepsin activity was eluted by an increasing concentration of NaCl in the same buffer. X shows the commencement of a convex gradient from 0.17 M- to 0.21 M-NaCl. At Y a linear gradient was applied from 0.21 M- to 1.0 M-NaCl. Fractions (10–11 ml.) were collected. —, E_{280} (total protein); O, E_{700} (peptic activity); $\square$, pH; ----, chloride concentration.

Fig. 7. Analysis of the pepsin peaks I-V eluted from the DEAE-cellulose column (see Fig. 6) by agar-gel electrophoresis at pH 5.0. ST is the standard marker of crystalline swine pepsin.

et al. (1963a). However, a spot was also located in peak V, with the mobility of pepsin 3. Since peak V was sharp and well separated from peak IV, this minor spot may represent some residual zymogen.

DISCUSSION

Agar-gel electrophoresis has become an important means of enumerating and characterizing the different pepsins produced by the human stomach.

Gastric juice can exhibit up to eight zones of proteolytic activity after electrophoresis in agar-gel at pH 5.0. Two of these zones, 4 and 6, are found not to represent unique pepsins. The former is possibly

a complex of a pepsin with an inhibiting peptide (Seijffers *et al.* 1964a), although this need not mean that its secretion into gastric juice is without physiological significance. The latter zone seems to represent a remaining zymogen. The available evidence indicates that the pepsin zones 1, 2, 3, 3a and 5 represent distinct and separate enzymes. The major pepsin, 3, and pepsin 5 are present in all normal gastric juices. The status of pepsins 1 and 2 is, however, less clear. These two pepsins only occasionally exist together in the same juice (in 8% of subjects). The zones were elongated and often weak, and, further, the actual locations of these two zones, relative to the standard, were more variable than the location of the other zones.

The zymograms for gastric juice published by Hirsch-Marie *et al.* (1965) exhibited activity that was designated as zones 1, 2, 3 and 4. Four zones had been noted previously for gastric-mucosal extracts by Kushner *et al.* (1964). The major pepsin found by both groups was at zone 2, with a single faster zone 1. The zone 3 described by Kushner *et al.* (1964) occupied a position equivalent to zone 4 in our zymograms. We found a single zone of activity for pepsin 5 at pH 5.0, but Hirsch-Marie *et al.* (1965) found two overlapping zones of activity, 3 and 4, at pH 5.6 in this region of the zymogram, with the faster-moving component displaying the greater activity. The zone 4 described by Kushner *et al.* (1964), was situated nearer the origin and this we consider to be equivalent to our zone 7 activity as located at pH 5.0. The position of the pepsins 3 and 4 described by Hirsch-Marie *et al.* (1965), in relation to our results, remains obscure.

Since our zone 7 was derived from an activity that did not migrate from the origin during electrophoresis at pH 5.0, we suggest that the isoelectric point is sufficiently high to be atypical of the pepsins.

Therefore we would exclude this proteinase from the pepsin group of enzymes.

Chromatography of gastric juice on Amberlite CG-50 gave essentially the same separation as that achieved by Richmond *et al.* (1957). The major pepsin, 3, was located in the first peak and the 'gastricsin' (Tang *et al.* 1959) in the second peak was identified with our pepsin 5. Fractionation of gastric juice on DEAE-cellulose with a salt gradient to 0.21M gave four peaks of activity, whereas Seijffers *et al.* (1963a) had located three, but they had taken steps to destroy any pepsin-inhibitor complexes. They showed that one of their peaks contained two pepsins, and in our separation peak II contained two pepsins. A fifth peak of activity was eluted by raising the salt concentration above 0.21M. This pepsin was apparently not eluted from the column by Seijffers *et al.* (1963a). Analysis of these peaks by agar-gel electrophoresis confirmed that more than one moved to zone 3 on the zymogram. That these pepsins have different biological properties has already been shown by Seijffers, Miller & Segal (1964b), and this strongly supports the view that the heterogeneity of zone 3 is not an artifact. Pepsin 5 was located in peak II and pepsin 1 in peak V. Chromatography of human pepsinogens on DEAE-cellulose has revealed a similar elution pattern (D. J. Etherington & W. H. Taylor, unpublished work).

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