STOMACH PROTEINASES SEPARATION, SPECIFICITY, AND CHANGES AFTER ADMINISTRATION OF SECRETION STIMULI

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The lyophilisate of human gastric juice was fractionated on Sephadex G-100 and the second proteolytically active fraction was separated further on DEAE-Sephadex A-50. The latter procedure afforded six proteolytically active fractions which were tested with various dipeptides as substrates. Whereas fraction No 6 cleaved exclusively acetyl-L-phenylalanyl-L-3,5-diiodotyrosine, the first four fractions cleaved formyl-L-tyrosyl-L-tyrosine and formyl-L-tyrosyl-L-phenylalanine. Fraction No 5 cleaved all three substrates to a lesser degree yet it possessed a relatively high activity when tested on hemoglobin. The character of the first four fractions is that of acid proteinases of the gastricsin type, the last fraction comprises proteinases of the pepsin type. The specific cleavage of synthetic substrates served as a basis for the development of a procedure permitting the determination of both groups of acid proteinases in native gastric juice; the ratio of both types of proteinases after administration of various stimuli of gastric secretion was examined.

During the past few years a large number of acid proteinases were obtained by fractionation of human gastric juice and the occurrence of their precursors in gastric mucosa was demonstrated. The fractionation of gastric proteinases has been effected by electrophoretic separation on various supports[1-2] or by ion exchange chromatography[5-9]. These problems have also been reviewed[10-12]. According to Samloff[11] all acid gastric proteinases can be classified with regard to their physico-chemical characteristics and site of origin of their precursors in gastric mucosa as belonging to two basic groups: proteinases of the gastricsin type or proteinases of pepsin type, according to the nomenclature of Tang and coworkers[5] who were the first to resolve these proteinases by chromatography on Amberlite IRC-50.

Acid gastric proteinases specifically cleave certain synthetic substrates of the dipeptide type and of other peptide types. One of the substrates most frequently used is acetyl-L-phenylalanyl-L-3,5-diiodotyrosine[13,14]. This substrate is specifically cleaved by acid proteinases of the pepsin type and is resistant to proteinases of the gastricsin type[15]. In this study we made a search for the most suitable substrate of acid gastric proteinases of the gastricsin type. We limited ourselves to the synthesis of substrates

of a similar type, replacing the hydrophobic N-acyl group by a hydrophilic formyl group.

EXPERIMENTAL

Fractionation of Gastric Proteinases

Human gastric juice was obtained after stimulation of its secretion by insulin (Spofa, Prague), administered intravenously in a dose of 0·25 U/kg body weight, by histamine (Spofa, Prague) administered subcutaneously in a dose of 0·023 mg/kg body weight, and by pentagastrin (Gastrodiagnost Merck), administered intravenously in a dose of 6 μg/kg body weight. In the experiments of the first series, mixed samples of gastric juice (after histamine stimulation) were dialyzed against water and lyophilized. In the experiments of the second series, the individual 15-ml portions of gastric juice, obtained from patients with peptic duodenal ulcer after stimulation by various agents, were filtered and both groups of proteinases were determined directly in native gastric juice. The removal of the gastric juice and its treatment were carried out at 0—4°C.

The lyophilisate of gastric juice (50 mg) was resolved by gel filtration on Sephadex G-100 (52 × 0·9 cm, 75 ml of 0·1M acetate buffer, pH 5·3) into three fractions. The first fraction was rich in hexoses which were determined by a modification of the orcinol method[16]. The presence of glycopeptides and polypeptides in the third fraction was detected by high voltage electrophoresis[17]. The second fraction, which contained acid gastric proteinases free of mucoproteins and peptides, was separated further on DEAE-Sephadex A-50 (12 × 0·9 cm). The fractionation was effected by elution by 1·6 l of 0·1M acetate buffer with an increasing salt gradient (0·05 to 0·35M-NaCl). Individual fractions were pooled, dialyzed against water, and lyophilized. Proteolytic activity according to Anson and Mirsky[18] and milk-clotting activity[19] were determined in individual fractions of the effluent from the DEAE-Sephadex column.

Determination of Gastric Proteinases

The acid gastric proteinases of the pepsin type were determined with acetyl-L-phenylalanyl-L-3,5-diiodotyrosine as substrate. Formyl-L-tyrosyl-L-tyrosine and formyl-L-tyrosyl-L-phenylalanine were used with acid gastric proteinases of the gastricsin type. Enzymatic hydrolysis of the synthetic substrates was assayed with 0·25 ml of 2 mM solution of the substrate in acetate buffer (pH 5·3) after the addition of 0·5 ml of effluent acidified to pH 2·5 by 0·3M-HCl. The mixture was incubated 48 h at 37°C. The C-terminal amino acids liberated were determined by the ninhydrin method according to Rosen[20]. Amino acid analyses of individual fractions from columns of Amberlite IRC-50 and DEAE-Sephadex were carried out with 2-mg portions of the lyophilisate of active fractions according to Spackmann and coworkers[21] by Dr A. Salvetová, Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. The fractions were analyzed electrophoretically in polyacrylamide[22] or agar[23] gel.

The determination of acid proteinases in native gastric juice was performed on 0·5 ml samples of the juice diluted with 0·05M-HCl (1 : 20), the samples were incubated with 0·15 ml of 2 mM substrate solution in 0·1M acetate buffer (pH 5·3) at 37°C. Acetyl-L-phenylalanyl-L-3,5-diiodotyrosine was used for the assay of gastric proteinases of the pepsin type and the mixture was incubated 3 h. The same quantity of formyl-L-tyrosyl-L-tyrosine or formyl-L-tyrosyl-L-phenylalanine was used for the determination of acid proteinases of the gastricsin type; the incubation was allowed to proceed for 5 h. At the end of the incubation 0·5 ml of 0·5M acetate buffer (pH 5·5) and 0·5 ml of the ninhydrin reagent[20] were added.

Synthesis of Substrates

N-*Formyl*-L-*tyrosyl*-L-*tyrosine ethyl ester* (I). A solution of N-formyl-tyrosine (2·3 g; 10 mmol) in dimethyl sulfoxide (15 ml) and chloroform (20 ml) cooled down to −5°C was treated with N-ethylpiperidine (1·4 ml) and sec-butyl chloroformate (1·4 ml). After 10 min of stirring and cooling tyrosine ethyl ester (2·10 g, 10 mmol) in dioxane (30 ml) was added to the reaction solution. The reaction mixture was set aside for 30 min at 0°C, then for 1 h at room temperature, and evaporated *in vacuo* afterwards. The dry residue was dissolved in ethyl acetate (30 ml) and water (10 ml), and the organic layer was washed with 1M-HCl, 5% sodium bicarbonate, and water, then dried and evaporated. The material obtained was crystallized from ethyl acetate (25 ml), ether (10 ml), and light petroleum (60 ml). The yield was 1·5 g (38%) of a product of m.p. 197—203°C. The sample for analysis was crystallized from tetrahydrofuran–tetrachloromethane–ether, m.p. 202—205°C. For $C_{21}H_{24}N_2O_6$ (400·4) calculated: 62·99% C, 6·04% H, 7·00% N; found: 62·97% C, 6·12% H, 7·14% N. $[\alpha]_D^{20}$ +23·8° (c = 0·2; acetic acid).

N-*Formyl*-L-*tyrosyl*-L-*tyrosine* (II). Compound *I* was stirred in 1M-NaOH (10 ml), 20 min at room temperature. Subsequently the solution was acidified with 2M-HCl and set aside for 4 h at 0°C. The product which had separated was filtered off, washed with cold water, and dried at room temperature. The yield was 0·62 g (83%) of a product of m.p. 202—204°C. The sample for analysis was recrystallized from acetic acid and light petroleum; the m.p. remained unaltered. For $C_{19}H_{20}N_2O_6$ (372·4) calculated: 61·28% C, 5·4% H, 7·52% N; found: 61·12% C, 5·52% H, 7·58% N. $[\alpha]_D^{20}$ +30·6° (c = 0·5; acetic acid).

N-Formyl-L-tyrosyl-L-phenylalanine methyl ester (*III*) was prepared by the carbodiimide method (using N,N′-dicyclohexyl carbodiimide and N-hydroxybenztriazole) in a yield of 73%. The sample for analysis was crystallized from 2-propanol and light petroleum; m.p. 175—177°C. For $C_{20}H_{22}N_2O_5$ (370·4) calculated: 64·85% C, 5·99% H, 7·56% N; found: 64·77% C, 6·21% H, 7·55% N. $[\alpha]_D^{20}$ +1·0 (c = 0·1; 50% acetic acid).

N-Formyl-L-tyrosyl-L-phenylalanine (*IV*) was prepared in analogy to compound *II* in a yield of 96%. The sample for analysis was crystallized from aqueous ethanol; m.p. 250—253°C. For $C_{19}H_{20}N_2O_5$ (356·4) calculated: 64·00% C, 5·64% H, 7·86% N; found: 64·10% C, 5·72% H, 7·63% N. $[\alpha]_D^{20}$ +4·5 (c = 0·4; methanol).

RESULTS AND DISCUSSION

The second fraction showing proteolytic activity, which had been obtained by separation of a human gastric juice lyophilisate on Sephadex G-100, was fractionated on Amberlite IRC-50. Two fractions[5], *i.e.* gastricsin and pepsin were obtained. When the same fraction from the Sephadex G-100 column was separated on DEAE-Sephadex A-50, 6 fractions with proteolytic activity were obtained (Fig. 1). The latter differed when judged by the criterions summarized in Table I. The amino acid analysis of the fractions from the Amberlite IRC-50 column gave values identical to those reported by Mills and Tang[23] (Table II). Similarly to Rajagopalan and coworkers[24] we too found the presence of lysine in the first and second fraction from the Amberlite IRC-50 column.

The amino acid analysis of the fractions which had been obtained by chromatography on DEAE-Sephadex of the proteolytically active material isolated from human gastric juice lyophilisate on Sephadex G-100, are given in Table III side by side

with the analyses of fractions from Amberlite IRC-50. The amino acid analyses of the fractions from DEAE-Sephadex showed an increase in aspartic acid and serine content from fraction one to fraction six whereas the glutamic acid content decreased from fraction one to fraction six. In comparison, the variations in the content of the

TABLE I

Comparison of Some Characteristics of Fraction I and VI of Human Gastric Proteinases Obtained by Chromatography on DEAE-Sephadex

The fractions were eluted by an increasing gradient of pH (4·0—4·6) and sodium chloride concentration (0·05M—0·35M). The proteolytic activity at pH 1·6 and 3·5 is expressed in mequiv of tyrosine. Inactivation by a change in pH was carried out at pH 8·0 (15 min incubation) and is expressed in per cent of activity determined at pH 5·4 (milkclotting activity).

Fraction	Emerging at pH	Proteolytic activity		Inactivation change in pH
		pH 1·6	pH 3·5	
I (gastricsin)	4·1	21·7	34·6	7·0
VI (pepsin)	4·6	62·7	37·2	91·6

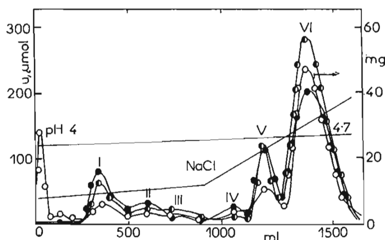

FIG. 1

Chromatography on DEAE-Sephadex A-50 of Human Gastric Juice Lyophilisate Free of Mucoproteins and Polypeptides on Sephadex G-100

The proteolytically active fractions are marked I—VI. The fractionation was effected by an increasing gradient of pH (4·0—4·7) and sodium chloride (0·05M—0·35M). ●, Milkclotting activity in units[19], ◐, proteolytic activity in μmol of Tyr. 10; ○, protein content according to Lowry *et al.*[34] (in mg). Chlorides were determined by visual mercurimetric titration in an acid medium with diphenylcarbazone as indicator.

TABLE II

Cleavage of Substrates by Individual Fractions of Human Gastric Proteinases Obtained by Chromatography on DEAE-Sephadex

The proteolytic activity (hemoglobin as substrate) at 1·6 is expressed in μmol of tyrosine. The synthetic substrates were cleaved at pH 2·5, the activity of the individual fractions is expressed in nmol of diiodotyrosine and in nmol of phenylalanine, respectively.

Substrate	Proteolytic Activity of Fraction					
	I	II	III	IV	V	VI
Hemoglobin	7·27	2·94	1·96	0·98	11·59	18·96
Ac-L-Phe-L.Tyr(I_2)	0	0	0	0	4·6	19·4
For-L-Tyr-L-Phe	10·45	7·38	7·18	3·72	0·80	0

TABLE III

Amino Acid Composition of Acid Proteinases Obtained by Chromatography on Amberlite IRC-50 Determined by the Method of Spackman and Coworkers[21]

The values indicate numbers of individual amino acid residues.

Amino Acid	Mills and Tang[23]		This Determination	
	Gastricsin	Pepsin	Gastricsin	Pepsin
Lysine	0	0	2	2
Histidine	1	1	1	1
Arginine	3	3	2	3
Aspartic Acid	26	40	28	40
Threonine	21	27	21	24
Serine	32	43	28	42
Glutamic Acid	39	31	26	29
Proline	17	19	15	21
Glycine	33	35	30	43
Alanine	18	18	18	19
Half-cystine	6	6	[a]	6
Valine	23	27	20	18
Methionine	5	5	4	5
Isoleucine	13	25	14	23
Leucine	25	22	22	25
Tyrosine	17	15	15	16
Phenylalanine	15	15	15	14

[a] Undeterminable quantity.

other amino acids are less striking. It appears that these changes also determine the character of substrate activity of the individual fractions. According to our findings the first four fractions show characteristics similar to Tang's gastricsin, the sixth fraction has features of an acid proteinase similar to Tang's pepsin[25]. The fractions obtained from Amberlite IRC-50 are not homogeneous. When subjected to polyacrylamide gel electrophoresis gastricsin yields 3–4 fractions, pepsin one major fraction and 3–4 minor fractions. The testing of the homogeneity of the individual fractions from DEAE-Sephadex by polyacrylamide gel electrophoresis is in progress in our laboratory.

Efforts to distinguish the individual fractions of the pepsin group and the gastricsin group by immunological or physico-chemical methods[11] have been unsuccessful

TABLE IV

Amino Acid Composition of Fractions Obtained by Chromatography on DEAE-Sephadex A-50

The proteolytically active fractions I, III, V and VI were obtained by fractionation on DEAE-Sephadex of the active fraction which had been isolated by gel filtration on Sephadex G-100 of the lyophilisate of human gastric juice. The analysis was carried out by the method of Spackman and coworkers[21]. The values indicate numbers of individual amino acid residues.

Amino Acid	Fraction			
	I	III	V	VI
Lysine	[a]	1	[a]	0
Histidine	[a]	1	[a]	1
Arginine	[a]	3	[a]	3
Aspartic Acid	26	38	39	40
Threonine	22	26	28	27
Serine	35	39	42	40
Glutamic Acid	42	35	33	32
Proline	17	19	19	18
Glycine	37	35	38	38
Alanine	22	18	20	18
Half-cystine	[a]	[a]	[a]	6
Valine	22	26	27	25
Methionine	5	3	8	4
Isoleucine	12	24	24	24
Leucine	25	22	22	23
Tyrosine	16	15	14	16
Phenylalanine	16	15	14	16

[a] undeterminable quantity.

so far. There is lack of informations on the number of acid proteinases in gastric juice, their role under physiological and pathological conditions, and on the number of their precursors in gastric mucosa. Seijffers and coworkers[27] were able to isolate by chromatography on DEAE-cellulose 3 pepsinogen fractions from human gastric mucosa yielding 4 pepsins after activation. Samloff[2] isolated 8 fractions by agar gel electrophoresis; of their number 7 showed the properties of pepsinogen. Mangla and coworkers[4] isolated 11 fractions by a similar technique. We have been able to fractionate the acid gastric proteinases on DEAE-Sephadex A-50 and to obtain several proteinases of the gastricsin type. Our efforts to separate the individual acid proteinases of the pepsin type have been futile so far.

We have employed the ability to cleave synthetic substrates by the fractions obtained by chromatography on DEAE-Sephadex of the proteolytically active part of human gastric juice lyophilisate as one of the characteristics of these fractions. Acetyl-L-phenylalanyl-L-3,5-diiodotyrosine is cleaved exclusively by the sixth fraction and to a lesser degree by the fifth fraction. Substrates formyl-L-tyrosyl-L-tyrosine and formyl-L-tyrosyl-L-phenylalanine are cleaved by fractions I–IV whereas fractions V and VI practically do not cleave these substrates or minimally (Table IV). This leads us to conclude that the latter two substrates are cleaved predominantly by acid proteinases of the gastricsin type (according to Tang[25]) or (according to Samloff's nomenclature[11]) by pepsins arising from pepsinogens of group II and localized in the whole stomach and in the duodenum[26].

TABLE V

Proteolytic Activity of Gastric Juice of Patients with Peptic Duodenal Ulcer after Various Secretion Stimuli

Histamine was administered subcutaneously in doses of 0·023 mg/kg body weight, pentagastrin intravenously in doses of 6 μg/kg of body weight, and insulin intravenously in doses of 0·25 U/kg body weight. The total proteolytic activity was determined by the method of Anson and Mirsky[18] (hemoglobin as substrate), the proteinases of the gastricsin group with For-L-Tyr-L-Tyr as substrate and proteinases of the pepsin group with Ac-L-Phe-L-Tyr(I_2) as substrate.

Secretion Stimulus	Number of Determinations	Proteolytic Activity		
		Hemoglobin mequiv Tyr ± S.E.	For-L-Tyr-L-Tyr μequiv Tyr ± S.E.	Ac-L-Phe-L-Tyr(I_2) μequiv Tyr ± S.E.
Histamine	21	$33{\cdot}04 \pm 5{\cdot}31^a$	$87{\cdot}93 \pm 17{\cdot}62^b$	$287{\cdot}72 \pm 45{\cdot}8^b$
Pentagastrin	18	$22{\cdot}43 \pm 4{\cdot}14^b$	$113{\cdot}44 \pm 29{\cdot}86$	$222{\cdot}85 \pm 34{\cdot}53^b$
Insulin	10	$62{\cdot}96 \pm 8{\cdot}00^{a,b}$	$188{\cdot}45 \pm 24{\cdot}90^b$	$600{\cdot}43 \pm 70{\cdot}45^{b,b}$

$^a p < 0{\cdot}025$, $^b p < 0{\cdot}005$.

The finding that formyl-L-tyrosyl-L-tyrosine and formyl-L-tyrosyl-L-phenylalanine are cleaved practically by acid proteinases of the gastricsin type only permitted us to carry out a quantitative determination of both groups of acid proteinases directly with the native gastric juice. We examined the ratio of secretion of both groups of enzymes after stimulation by various agents. The secretion was examined with 49 patients with a peptic duodenal ulcer (average age 36 years). The secretion of hydrochloric acid and the activities of the individual proteinases are given in Table V. Like other authors[28-30] neither we found any differences in the action of pentagastrin and histamine on the volume of gastric juice secreted and on the degree of secretion of hydrochloric acid. Higher values were obtained after the administration of insulin yet the difference is not statistically significant (see also[32]). Compared to the remaining stimuli insulin significantly increases the secretion of pepsin[32].

The secretion of the individual groups of acid proteinases has not been investigated so far with a larger number of patients with disorders of the digestive tract. Our results indicate that the action of different secretion stimuli on various groups of proteinases is different. Insulin stimulates above all acid gastric proteinases belonging to the pepsin group whereas acid gastric proteinases of the gastricsin group are stimulated to the relatively highest degree by pentagastrin. This conclusion is obvious from the ratio of the total release of the individual acid proteinases[33] to the total release of hydrochloric acid (Table VI).

TABLE VI

Ratio of Secretion of Total Acid Proteinases of Human Gastric Juice and of Proteinases of Pepsin and Gastricsin Group to Secretion of Hydrochloric Acid in Patients with Peptic Duodenal Ulcer

Secretion Stimulus	Total Proteinases: HCl ±S.E.	Pepsin: HCl ±S.E.	Gastricsin: HCl ±S.E.
Histamine	1.51 ± 0.13^{a}	$13.40 \pm 1.22^{a,b}$	4.57 ± 0.97
Pentagastrin	1.10 ± 0.10^{a}	$10.00 \pm 1.26^{a,b}$	5.31 ± 1.19
Insulin	$2.86 \pm 0.51^{a,a}$	$27.80 \pm 0.51^{b,b}$	3.82 ± 0.33

^{a} $p < 0.05$, ^{b} $p < 0.005$.

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