Promotion of Activation of Pepsinogen by Polyanions Including RNA and Sulfated Mucosubstances[1] (34579)

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The inhibitory effect of polyanions on the hydrolysis of substrates by pepsin is a phenomenon which has been reported extensively (1–5). Little attention, however, has been given to the effects of polyanions upon the activation of the zymogen, pepsinogen. The activation of pepsinogen was represented schematically by Herriott (6, 7) as follows:

$$\text{Pepsinogen} \longrightarrow \text{Pepsin inhibitor compound} \underset{>\text{pH } 5.4}{\overset{<\text{pH } 5.4}{\rightleftharpoons}} \text{Pepsin} + \text{inhibitor.}$$

Later, it was shown by Van Vunakis and Herriott (8) that a basic peptide is split off from the zymogen during the activation process.

At the outset of this investigation, it was not possible for us to predict whether polyanions would promote or retard the activation process. On the one hand, it was conceivable that the polyanions could complex with pepsinogen preventing the cleavage reaction and the separation of the basic peptide, but, on the other hand, it also seemed reasonable to think that the polyanions could facilitate the cleavage reaction and the detachment of the basic peptide from the enzyme by combining with the former and removing the reaction product.

Preliminary observations by Pamer (9) indicated that chondroitin 4-sulfate promotes the activation of pepsinogen. Shortly after the submission and acceptance of Pamer's doctoral thesis and the completion of our research on this subject, Anderson (10) communicated his observations that heparin and chondroitin 4-sulfate activate pepsinogen. We report here that the polyanions ribonucleic acid (RNA), chondroitin 4-sulfate, heparin, and a sulfated glycoprotein fraction from human gastric juice promote the activation of pepsinogen.

Methods. Activation. Pepsinogen (porcine pepsinogen, PG7JA, Worthington Biochemical Corp., Freehold, New Jersey) was dissolved in glass-distilled water giving a solution containing 1 mg/ml of the zymogen. To 1 ml of the pepsinogen solution were added 2 ml of buffer of the desired pH, (0.05 *M* acetate buffer, pH 3.5, 4.0, 4.5, or 5.0, or 0.05 *M* phosphoric acid–phosphate buffer, pH 3.0) and 1 ml of an aqueous solution of polyanion (5 mg/ml). Polyanions used were yeast ribonucleic acid (RNA 8KA, Worthington Biochemical Corp., Freehold, New Jersey), chondroitin 4-sulfate (Institute for Arteriosclerosis Research, Los Angeles, California), heparin (Abbot Laboratories, North Chicago, Illinois), canine gastric sulfated glycoprotein Fraction IIIB isolated by Pamer *et al.* (11) from dog gastric mucosa and a sulfated glycoprotein fraction I isolated from human gastric juice. The latter glycoprotein fraction contained 4 % (w/w) sulfate and was isolated by the procedure described by Palmer *et al.* for isolating Fraction I of the canine sulfated glycoproteins. The resulting solution was incubated for 10 min in a water bath at 28°. The activation mixture was removed from the bath, and the solution was rendered alkaline immediately by addition of 2 ml of 0.05 *M* Na_2HPO_4 and sufficient 0.04 *N* NaOH to give pH 8.5. Then the solution was kept at room temperature for 30 min. The alkalinization inactivated any pepsin initially present or formed during the activation process. These conditions for inactivation of pepsin were tested on a solution containing 2600

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TABLE I. Effect of pH on the Promotion of Pepsinogen Activation by RNA.[a]

pH during activation	Experimental[b] (units/mg)	Control[c] (units/mg)	Increase in activation by polyanions (%)[d]
3.0	2 (± 0.2)	13 (± 3)	0.5
3.5	13 (± 4)	843 (± 29)	47
4.0	166 (± 10)	2450 (± 86)	1522
4.5	2250 (± 73)	2530 (± 91)	400
5.0	2630 (± 82)	2610 (± 77)	0

[a] Results are expressed in terms of units of potential pepsin activity remaining in the activation mixture after activation and alkalinization per milligram of pepsinogen. Data presented are for the mean and standard deviation of triplicate determinations.

[b] RNA was present during activation of pepsinogen in experimental tubes.

[c] RNA was absent during activation of pepsinogen in control tubes; RNA was added after alkalinization of activation mixtures.

[d] $[(\Delta E - \Delta C)/\Delta C] \times 100 =$ percentage increase in activation in presence of polyanion; $\Delta E =$ potential pepsin activity of standard pepsinogen (2600 units/mg)—potential pepsin activity of pepsinogen remaining in experimental tubes; $\Delta C =$ potential pepsin activity of standard pepsinogen (2600 units/mg)—potential pepsin activity of pepsinogen remaining in control tubes.

units/ml of porcine pepsin (PM8JB, Worthington Biochemical Corp.) and gave complete, irreversible inactivation. Tubes containing pepsinogen and buffer, but not containing polyanion during the activation process, served as controls; after activation, the control tubes were adjusted to pH 8.5 and then polyanion was added.

After the alkalinization step, solutions were acidified with 0.1 *N* HCl to pH 2. The solution was kept at room temperature for 30 min and then diluted with 0.01 *N* HCl to 25 or 50 ml for the assay described under Hydrolysis.

Hydrolysis. An aqueous 2 % hemoglobin substrate (bovine hemoglobin, Worthington Biochemical Corp.) was used at pH 1.6 as described by Anson (12). One ml of a suitably diluted solution of activation mixture was added to 5 ml of the hemoglobin substrate and incubated at 37° for 10 min. The hydrolysis of substrate was terminated by the addition of 10 ml of 5% trichloroacetic acid (TCA). The resulting suspension was filtered through Whatman No. 50 paper, and the absorbancy of the filtrates at 280 mμ was determined spectrophotometrically (13). All assays were performed in triplicate.

One unit of activity is equal to the absorbancy at 280 mμ of TCA-soluble hydrolysis products of 0.001/min. Assay results were expressed as units of potential pepsin activity remaining in the activation mixture after activation and alkalinization/mg of pepsinogen. Assays also were performed on pepsinogen and pepsin which were not exposed to the added polyanions during the activation or digestion stages.

Results. A tabulation of the proteolytic activity remaining per milligram of pepsinogen after initial activation at various pH values in the presence of RNA and subsequent alkalinization is provided in Table I. These activities are compared with the activities remaining in control tubes where initial activation occurred in the absence of RNA. The promotion of activation is most pronounced at pH 3.5–4.0 as shown in studies with RNA. At pH 4.0, a 1522 % increase in promotion of activation occurs in the presence of RNA. Under the conditions employed by us, 28° for 10 min, little, if any, activation of pepsinogen is discerned in the absence of RNA. At pH 3.0, activation by H^+ and by pepsin becomes predominant, and the promotion by polyanion is barely, if at all, discernible.

A variety of polyanions, including chondroitin 4-sulfate, RNA, heparin, and a crude sulfated glycoprotein fraction isolated from gastric juice promoted the activation of pepsinogen. The activity remaining after activation of pepsinogen at pH 4.0 in the presence of various polyanions and subsequent alkalin-

TABLE II. Effect of Various Polyanions on Activation of Pepsinogen at pH 4.0.[a]

Polyanion	Experimental (units/mg)	Control (units/mg)	Increase in activation by polyanions (%)
Chondroitin 4-sulfate	1761 (± 57)	2495 (± 81)	670
Heparin	10 (± 1.1)	2440 (± 73)	1519
RNA	166 (± 10)	2450 (± 86)	1522
C-Fraction IIIB[b]	2474 (± 84)	2490 (± 77)	14
HGJ-Fraction I[c]	1710 (± 53)	2483 (± 35)	661

[a] Results expressed as for Table I.

[b] C-Fraction IIIB = sulfated glycoprotein fraction IIIB from canine gastric mucosa.

[c] HGJ-Fraction I = crude sulfated glycoprotein fraction from human gastric juice.

ization is given in Table II. The order of effectiveness in promotion of the activation of pepsinogen by polyanions was heparin ≈ RNA > chrondroitin 4-sulfate ≈ fraction I from human gastric juice.

Conventional assays of standard pepsinogen and pepsin, without recourse to the preliminary acidification and alkalinization, were performed in the presence of 100–200 μg of RNA or chondroitin 4-sulfate/5 ml of hemoglobin substrate and gave 2600 units/mg of pepsinogen and 2550 units/mg of pepsin. These values were in good agreement with the values of 2675 units/mg of pepsinogen and 2625 units/mg of pepsin obtained for those preparations when assayed in the absence of added polyanions.

The amount of polyanion present, 100–200 μg per mg of hemoglobin substrate, in both experimental and control assay tubes, was insufficient to inhibit hydrolysis of hemoglobin, and accordingly, the effect reported here was not generated at the stage of hydrolysis of substrate. Moreover, the addition of polyanions to control tubes after activation and alkalinization insured that the sole difference between experimental and control tubes was the presence of the added polyanion during the initial activation period.

Discussion. Perlman (14) has shown that the configuration of pepsinogen is stabilized by the side-chain interaction of a predominantly electrostatic nature between the basic amino acid residues of the peptide segment that is released during activation and some of the dicarboxylic acids of the protein moiety. It is believed that a transient complex is formed between the basic peptide, after it is cleaved from the protein, and the residual protein of pepsin. Dissociation of this complex must occur for the enzyme to assume the configuration required for activity. We interpret the promotion of pepsinogen activation by polyanion as resulting from an interaction of the basic peptide with the added polyanion, thus disrupting the pepsin–inhibitor complex. With reference to this mechanism, it is interesting to recall the observation of Katchalsky *et al.* (15) that polylysine formed a complex with pepsin, rendering the pepsin inactive; they observed that this complex was analogous to the pepsin–inhibitor complex formed during activation of pepsinogen. Enzyme activity was restored by addition to the system of heparin which in turn complexed with polylysine.

It was wholly expected that heparin would be the most effective compound in the group of polyanions promoting activation of pepsinogen since heparin contains sulfate groupings of low p*K*a and exhibits the highest charge density. Surprisingly, RNA, which is of lower charge density and contains anionic groups of higher p*K*a than chondroitin 4-sulfate, was more effective than chondroitin 4-sulfate in promoting the activation of pepsinogen. Studies of interactions of RNA or chondroitin 4-sulfate with cationic detergents such as cetylpyridinium or cetyltrimethylammonium bromide and determinations of electrophoretic mobilities have shown that RNA is a weaker polyanion than chondroitin 4-sulfate (16). Thus it appears that charge density is not the sole factor affecting the interaction of the inhibitor with polyanion. The conformation and molecular weight of

the polyanion probably also play an important part in this interaction.

The human sulfated glycoprotein fraction promoted activation of pepsinogen though the canine preparations did not. Although it is possible that the differences between these glycoproteins in charge distribution and stereochemistry may account for this difference in behavior, it is also possible that the human fraction I was active in promoting activation owing to the presence in this fraction of contaminating RNA and of sulfated mucopolysaccharides. Additional studies comparing sulfated glycoproteins from human and canine gastric mucosa and secretions must await the availability of purified preparations from each of these sources.

Under what conditions will the promotion of pepsinogen activation by polyanions assume a physiological significance? While the answer to this question is not known at present, it is conceivable that this phenomenon may assume importance in conditions associated with altered permeability of the mucosa and consequent back-diffusion of H^+ into the chief cells (17, 18). Where the resulting increase in $[H^+]$ in the chief cells is insufficient in and of itself to activate pepsinogen at a rapid rate, interaction of pepsinogen with polyanion at that pH may afford a rapid activation of pepsinogen. The resulting pepsin could contribute to cellular disruption by hydrolyzing cellular proteins.

Gerard *et al.* (19) have shown that a chondroitin sulfate-like substance is present in the chief peptic cells of the dog stomach. Gerard *et al.* (20) and De Graef *et al.* (21) showed that this substance is discharged together with pepsinogen upon stimulation by urecholine. Sulfated glycoprotein, though present in the superficial epithelium and crypt cells of the fundus and the crypt cells of the antrum, were absent from the chief cells (19). Thus, interactions between pepsinogen and chondroitin sulfate (but not with sulfated glycoprotein) conceivably may occur in the chief cells.

Anderson (10) suggested that gastric sulfated glycosaminoglycans, when present in restricted quantities in the stomach chief cells, could participate in this "pathological" action, but when present in larger amounts would offset the pathological consequences by inhibiting hydrolysis. We agree with this statement and believe that it could be made more general by including RNA as a polyanion which could participate in the promotion of activation of pepsinogen. It is conceivable that premature activation of pepsinogen by polyanions could call forth the elaboration of larger amounts of sulfated glycosaminoglycan which would by combination with cellular proteins inhibit the hydrolysis of these proteins.

Since pepsinogen is elaborated in the chief cells in the form of zymogen granules surrounded by a membrane, it will be of interest to determine if this membrane shields pepsinogen from interaction with polyanion, especially in the range of pH 4–4.3.

Summary. Heparin, RNA, a sulfated glycoprotein from human gastric juice, and chondroitin 4-sulfate promote activation of pepsinogen. Heparin and RNA are about twice as effective as the sulfated glycoprotein and chondroitin 4-sulfate in promoting activation. The promotion of activation of pepsinogen is pH dependent. The pH for maximum promotion of activation by RNA was approximately pH 4.0.

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