Partial Characterization of Human Pepsin I, Pepsin IIA, Pepsin IIB, and Pepsin III*

MAX J. SEIJFFERS,† LEON L. MILLER, AND HARRY L. SEGAL

From the University of Rochester School of Medicine and Dentistry, Rochester, New York

Received November 12, 1963; revised April 24, 1964

Some physicochemical characteristics of four human pepsins isolated in small amounts from a *single* human stomach have been studied. This inherent technical restriction allowed only limited and empirical comparisons; despite this, significant differences were observed and support the view that the four chromatographically distinct pepsins also differ somewhat in certain other physicochemical properties. Under the experimental conditions used, two separate *p*H optima are present in activity-versus-*p*H curves for proteolysis of bovine hemoglobin by pepsin I (at *p*H 1.4 and 3.0) and for proteolysis of bovine serum albumin by pepsin I (at *p*H 1.2 and 3.3), pepsin IIA (at *p*H 1.5 and 3.2), pepsin IIB (at *p*H 1.5 and 3.0), and pepsin III (at *p*H 1.5 and 3.0). Under comparable experimental conditions a single *p*H optimum between *p*H 2.0 and 3.0 is present in activity-versus-*p*H curves for proteolysis of bovine hemoglobin by pepsins IIA, IIB, and III. Inactivation by alkali at *p*H 7.0 (pepsins IIA, IIB, and III) and at *p*H 7.4 (pepsin I) is first order with time over a 20-minute period. Resistance to alkali decreases in the following order: pepsin I, pepsin IIB, pepsin IIA, pepsin III. Inactivation by acid (*p*H 2.4) at 65° (pepsins IIA, IIB, and III) and at 62° (pepsin I) is first order with time over a 20-minute period. Resistance to acid decreases in the following order: pepsin IIB, pepsin IIA, pepsin III, pepsin I. Thus pepsin I differs significantly from other human pepsins; it is much more alkali resistant and more labile at *p*H 2.4. Inactivation by heat (*p*H 5.4, 62°) is first order with time over a 20-minute period. Resistance to heat decreases in the following order: pepsin IIB, pepsin I, pepsin IIA, pepsin III. At *p*H 2.4 bovine hemoglobin protects pepsins IIA, IIB, and III to a certain degree against heat inactivation. Human pepsins differ in relative substrate specificity toward bovine hemoglobin, bovine serum albumin, and milk protein.

The chromatographic separation of three pepsinogen fractions, which have been called pepsinogen I, pepsinogen II, and pepsinogen III, from human fundic mucosal extracts has been reported (Seijffers *et al.*, 1963b). Acidification of these pepsinogen fractions and subsequent chromatography has shown that it is possible to isolate four pepsin fractions: pepsin I from pepsinogen I, pepsin III from pepsinogen III, and pepsin IIA and pepsin IIB both from pepsinogen II. Human pepsin IIB and pepsin III were shown to have the same chromatographic mobility on DEAE-cellulose. However, indirect evidence makes it unlikely that pepsin IIB and pepsin III are identical (Seijffers *et al.*, 1963c). A study of their properties, as reported here, provides direct evidence for this view.

Apart from chromatographic behavior, no comparison of physicochemical data pertaining to these four pepsins has been reported. This report describes an empirical comparison of certain physicochemical properties made within the technical restrictions imposed by the small amounts of material available from a *single* human stomach. The limited results affirm the view that the four pepsins are physicochemically different.

METHODS AND RESULTS

Separation of Pepsins.—Chromatographic separation of pepsinsogens and pepsins employing DEAE-cellulose has been described in detail (Seijffers *et al.*, 1963b,c). To obtain pure pepsin solutions for further study the following successive fractionations were performed: Fundic mucosa extract from a single stomach specimen[1] was fractionated on a DEAE-cellulose column to yield pepsinogens I, II, and III. Rechromatography of each pepsinogen fraction was performed to obtain solutions of pure pepsinogen fractions. Each pepsinogen solution was thereafter acidified with 2 N HCl to *p*H 2, let stand for 5 minutes, then brought to *p*H 4 with 2 N sodium acetate, let stand for 1 hour, then dialyzed and subjected to chromatography as previously described (Seijffers *et al.*, 1964b). Pepsins IIA and IIB obtained from fractionation of acidified pepsinogen II solution were refractionated to yield pure pepsin IIA and pure Pepsin IIB solutions. The final purified pepsin solutions obtained by pooling of eluate fractions were dialyzed against 0.001 M sodium acetate buffer, *p*H 5.4, and stored at 4°. To obtain results in the linear range of the method for assay of proteolytic activity these pepsin stock solutions were suitably diluted with 0.001 M acetate buffer, *p*H 5.4, before use in experiments.

Assay of Proteolytic Activity with Acid Hemoglobin.—This assay was based on the method of Anson and Mirsky (1932). Hemoglobin powder (Fisher Scientific Co.) was heated at 60° for 3 hours to destroy inherent proteolytic activity. Substrate for assay of proteolytic activity was acid hemoglobin consisting of 1 part 0.29 N HCl solution and 4 parts of 2.5% (w/v) hemoglobin. Typically, 5.0 ml of acid hemoglobin and 1.0 ml of solution for assay were mixed after temperature equilibration and incubated at 37° for 60 minutes along with appropriate blanks. By using HCl solutions of appropriate normality (0.18–0.19 N) for *p*H adjustments, *p*H

* Requests for reprints are to be sent to: Dr. Harry L. Segal, Department of Medicine, The University of Rochester School of Medicine and Dentistry, 260 Crittenden Blvd., Rochester, New York 14620. This study was supported by a U. S. Public Health Service grant (CS-9546 (C-4)) and a U. S. Public Health Service training grant (5177-06).

† Present address: Department of Medicine A, Hadassah Hebrew University Hospital and Medical School, Jerusalem, Israel.

[1] Fundic mucosa was obtained from a stomach specimen of a white male, aged 64 years, subjected to partial gastrectomy following repeated hematemesis. Two antral ulcers were present on pathological examination. The preparation of fundic mucosal extracts has been described (Seijffers *et al.*, 1963b).

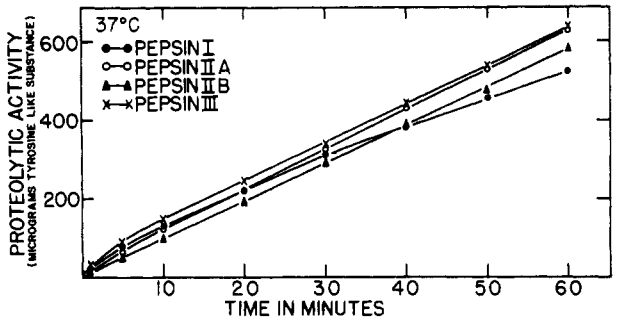

FIG. 1.—Progress of proteolysis of bovine hemoglobin at 37° at *p*H 2.4–2.5 by human pepsins.

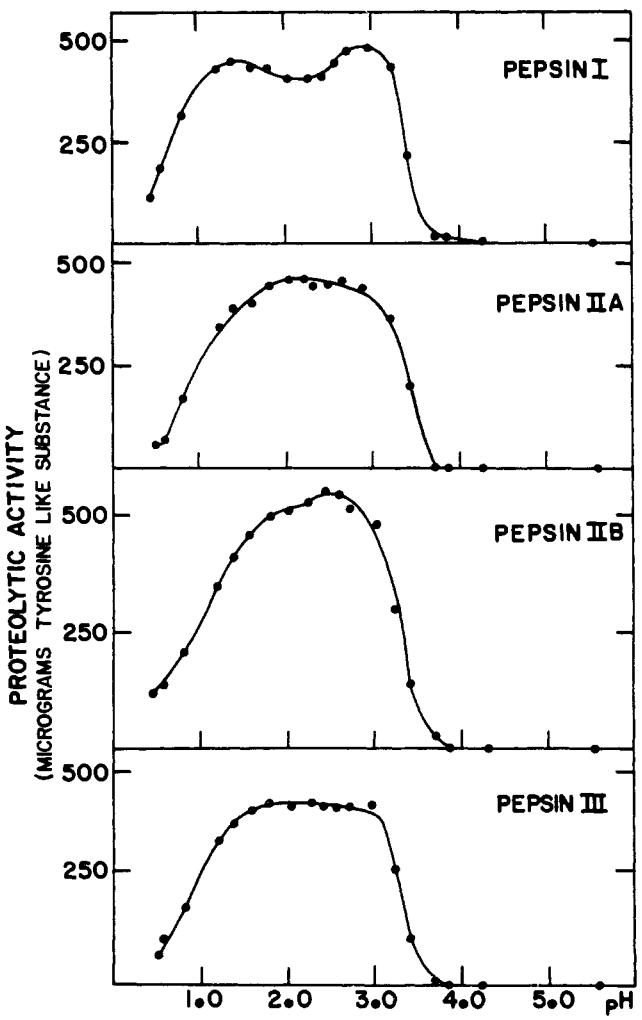

FIG. 2.—Proteolytic activity–versus–*p*H curves for proteolysis of bovine hemoglobin at 37° by human pepsins.

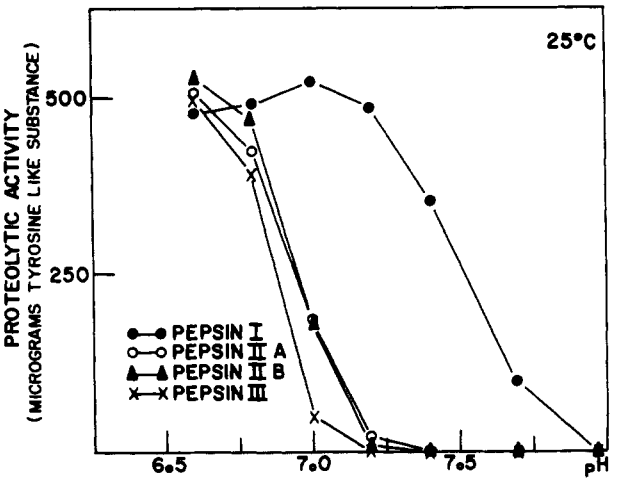

FIG. 3.—Alkali inactivation of human pepsins as a function of *p*H at 25°.

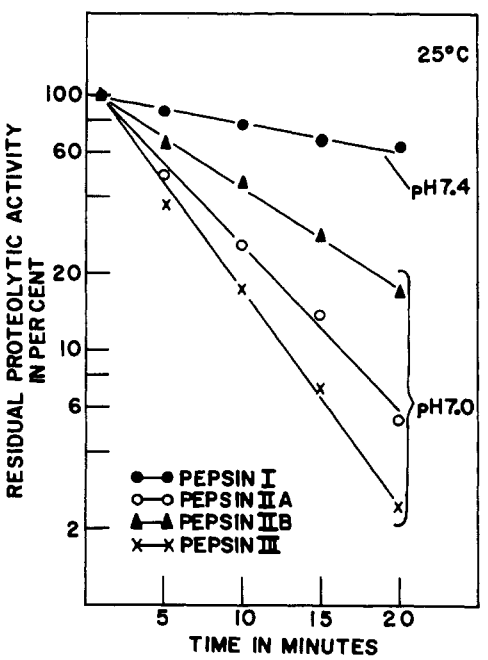

FIG. 4.—Time course of alkali inactivation of human pepsins at 25°. Semilogarithmic plot of per cent residual proteolytic activity. Inactivation of pepsin I is over a 20-minute period, significant only at *p*H 7.4; for this reason progress of alkali inactivation of pepsin I is followed at *p*H 7.4, instead of *p*H 7.0.

of incubation mixtures was set between 2.4 and 2.5 in all experiments unless otherwise qualified. In this *p*H range proteolytic activity does not vary significantly. The reaction was terminated by addition of 9 ml 0.3 M trichloroacetic acid. A tyrosine standard consisting of 5 ml acid hemoglobin and 1 ml of 500 μg/ml tyrosine was incubated during the same period of time and served, after the addition of 9 ml trichloroacetic acid as standard. The resulting trichloroacetic acid supernatants were assayed for tyrosinelike substance with Folin's phenol reagent (Miller *et al.*, 1957) and optical densities were measured with a Coleman Junior spectrophotometer at 535 mμ. Results were expressed as micrograms tyrosinelike substance released by 1 ml of solution for assay from 5 ml acid hemoglobin during 60 minutes' incubation at 37° in reference to the tyrosine standard of 500 μg tyrosine unless otherwise qualified. All determinations were performed in duplicate. Unless otherwise qualified the term "proteolytic activity" in this report refers to proteolytic activity of pepsins at *p*H 2.4–2.5 with acid hemoglobin as substrate, measured in terms of tyrosinelike substance.

*Time Course of Proteolysis of Bovine Hemoglobin (37°, p*H *2.4–2.5).*—The extent of proteolysis by human pepsins over a 60-minute period was followed by assay of tyrosinelike substance released from 5 ml acid hemoglobin 1, 5, 10, 20, 30, 40, 50, and 60 minutes after addition of 1 ml pepsin solution. The curves depicted in Figure 1 show that pepsin solutions releasing not more than about 600 micrograms tyrosine-like substance from bovine hemoglobin after 60 minutes incubation at 37°, produce linear progression of proteolysis (pepsin

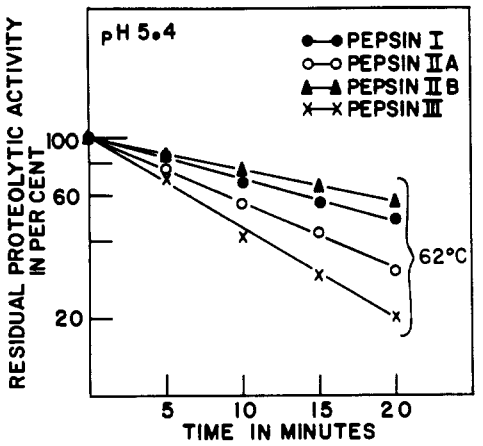

FIG. 5.—Time course of heat inactivation of human pepsins at *p*H 5.4 (62°). Semilogarithmic plot of per cent residual proteolytic activity.

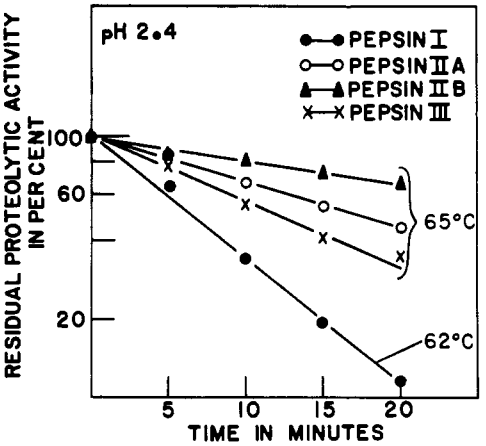

FIG. 6.—Time course of heat inactivation of human pepsins at *p*H 2.4. Semilogarithmic plot of per cent residual proteolytic activity. Inactivation of pepsin I is so rapid at 65° that results of inactivation at 62° are plotted instead.

IIA, IIB and III) or only a slight departure from linearity (pepsin I).

Determination of Optimal pH of Proteolysis of Bovine Hemoglobin.—In a typical analysis 4 ml of bovine hemoglobin and 1 ml from each of a series of dilute HCl solutions (ranging from 2.4 to 0.03 N) were mixed and allowed to equilibrate at 37°, and 1 ml of a human pepsin solution was added. Incubation mixtures were, along with appropriate blanks, incubated for 60 minutes at 37°, after which the reaction was terminated with trichloroacetic acid. Assay for tyrosinelike substance was performed as described above. The *p*H of incubation mixtures (4 ml bovine hemoglobin, 1 ml HCl, and 1 ml pepsin solution) was determined in parallel aliquots at beginning and end of 60 minutes' incubation. The average *p*H obtained from the *p*H at beginning and end of the 60-minute incubation period did not differ by more than 0.1 *p*H unit from either. This average value was presumed to represent *p*H of incubation and is the value plotted in Figure 2.

Alkali Inactivation at Various pH.—Phosphate buffers, 0.1 M, (*p*H 0.5–8.0), were equilibrated at 25° and one volume of pepsin solution (25°) was added to one volume of phosphate buffer. After 10 minutes' incubation at 25°, residual proteolytic activity of these mixtures was assayed with acid hemoglobin (*p*H of 5 ml acid hemoglobin plus 1 ml assay mixture was 2.3–2.6) at 37°. The *p*H of phosphate buffer–pepsin solution mixture was determined in parallel aliquots. Figure 3 shows how Pepsins IIA, IIB, and III lose significant proteolytic activity at a *p*H exceeding 6.8, and are completely inactivated at *p*H 7.2. At the latter *p*H pepsin I is stable up to 10 minutes, and loss of proteolytic activity occurs only at a higher *p*H.

Time Course of Alkali Inactivation at 25° at pH 7.0.—This time course was determined by mixing 0.1 M phosphate buffer, *p*H 7.0, with pepsin solution (pepsins IIA, IIB, and III were employed) as described (Fig. 4). Residual proteolytic activity was assayed with acid hemoglobin by withdrawing aliquots 1, 5, 10, 15, and 20 minutes after mixing. Progress of inactivation of pepsin I was similarly followed at *p*H 7.4 (0.1 M phosphate buffer, *p*H 7.4). Residual proteolytic activity is plotted semilogarithmically as percentage of proteolytic activity after 1-minute alkali inactivation versus time of alkali inactivation. Inactivation of pepsin I at *p*H 7.4 and of the three other pepsins at 7.0 follows an apparent first-order reaction with time. Pepsins III, IIB, and I, in this order, have increasing resistance to alkali.

Heat Inactivation at pH 5.4.—Aliquots of pepsin solution were, after incubation for 5, 10, 15, and 20 minutes at 62°, transferred to a water bath at 0° to effect rapid cooling. Assay for residual proteolytic activity was performed on these aliquots and on an unheated aliquot ("0 minutes' heat inactivation") as described. A similar experiment was performed at 65°. Figure 5 shows percentage residual proteolytic activity plotted semilogarithmically versus time of heat inactivation in minutes. Progress of inactivation is apparently first order. After 10 minutes' incubation at 65° and *p*H 5.4 all four pepsins had lost more than 80% of their proteolytic activity.

Heat Inactivation at pH 2.4.—Equal parts of pepsin solution (in 0.001 M acetate buffer, *p*H 5.4) and 0.012 N HCl were mixed (*p*H of mixture, 2.4) and aliquots were immediately transferred to a water bath set at 62°. Heat inactivation and assay for residual proteolytic activity were thereafter performed exactly as described previously for heat inactivation at *p*H 5.4, and results are shown in Table I. A similar experiment was performed at 65° employing pepsins IIA, IIB, III. As seen in Figure 6, pepsin I stands out by its relative heat lability at *p*H 2.4, in contrast with its relative heat resistance at *p*H 5.4 (cf. Fig. 5). The three other pepsins are conversely more heat resistant at *p*H 2.4 than at *p*H 5.4 (*vide supra*).

TABLE I
HEAT INACTIVATION AT 62° (*p*H 2.4)[a]

Time (min)	Residual Proteolytic Activity (%) Pepsin IIA	Pepsin IIB	Pepsin III
0	100	100	100
5	92	96	95
10	83	94	91
15	81	87	81
20	76	82	77

[a] Heat inactivation of pepsin I at 62° and *p*H 2.4 is depicted in Figure 6.

Effect of Temperature on Proteolysis of Bovine Hemoglobin at pH 2.4–2.5.—Incubation mixtures consisting of 5 ml acid hemoglobin and 1 ml pepsin solution were placed at 62° and extent of proteolysis determined after 1, 5, 10, 20, 30, 40, and 60 minutes by measurement of tyrosinelike substance released. The same

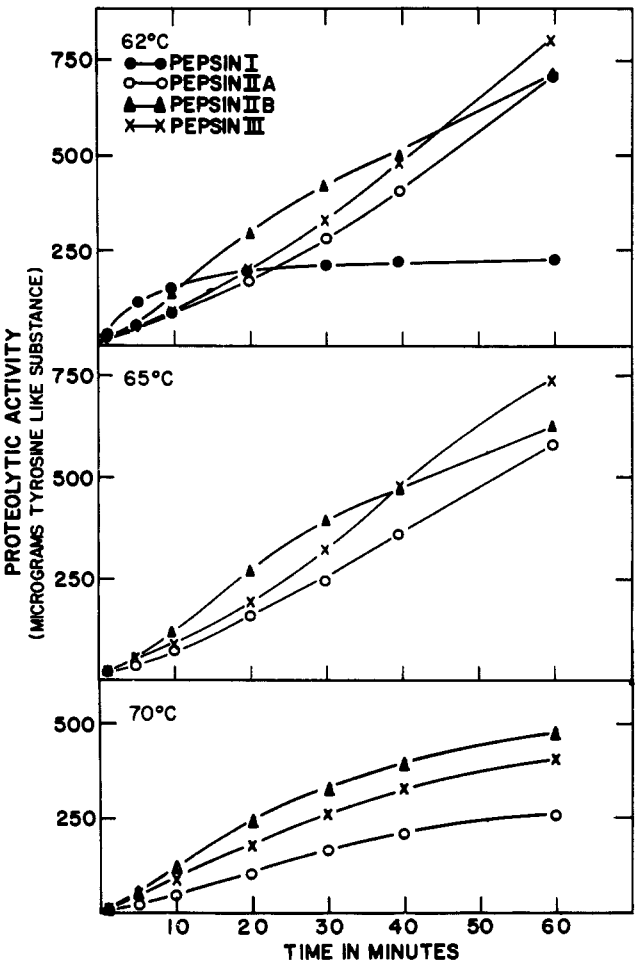

FIG. 7.—Progress of proteolysis of bovine hemoglobin by human pepsins at *p*H 2.4–2.5 from above downward at 62°, 65°, 70°, respectively. Pepsin I is already inactivated at 62° and has therefore not been studied at higher temperature.

experiment using pepsins IIA, IIB, and III solution was performed at 65° and 70° as well. Figure 7 reveals that pepsin I, after 20 minutes' incubation at 62°, is almost completely inactivated, and pepsin IIB loses some proteolytic activity at 62°. Proteolytic activity curves of pepsins IIA and III have at 62° an upward curvature and do not reveal gross heat inactivation of these pepsins. Pepsins IIA and IIB are inactivated at higher temperature (Figure 7B,C), at about the same rate.

Determination of Optimal pH for Proteolysis of Bovine Serum Albumin.—In a typical analysis, 4 ml of 0.5% BSA (bovine serum albumin fraction V, Armour & Co.)[2] and 1 ml from a series of dilute HCl solutions (ranging from 2.8 to 0.006 N) were mixed and allowed to equilibrate at 37°, and 1 ml pepsin solution was added. These incubation mixtures which were incubated for 60 minutes at 37° provide sufficient substrate for linear progression of proteolysis. The reaction was terminated by addition of 9 ml 0.3 M trichloroacetic acid. Assay for tyrosinelike substance was performed as described for assay of proteolysis of bovine hemoglobin. The *p*H of incubation mixtures was determined in parallel aliquots at beginning and end of 60 minutes' incubation. The average *p*H obtained from the *p*H at beginning and end of the 60-minute incubation did not differ from either by more than 0.1 *p*H unit, and is the *p*H plotted in Figure 8 which portrays the results.

[2] Abbreviation used in this work: BSA, bovine serum albumin fraction V, Armour & Co.

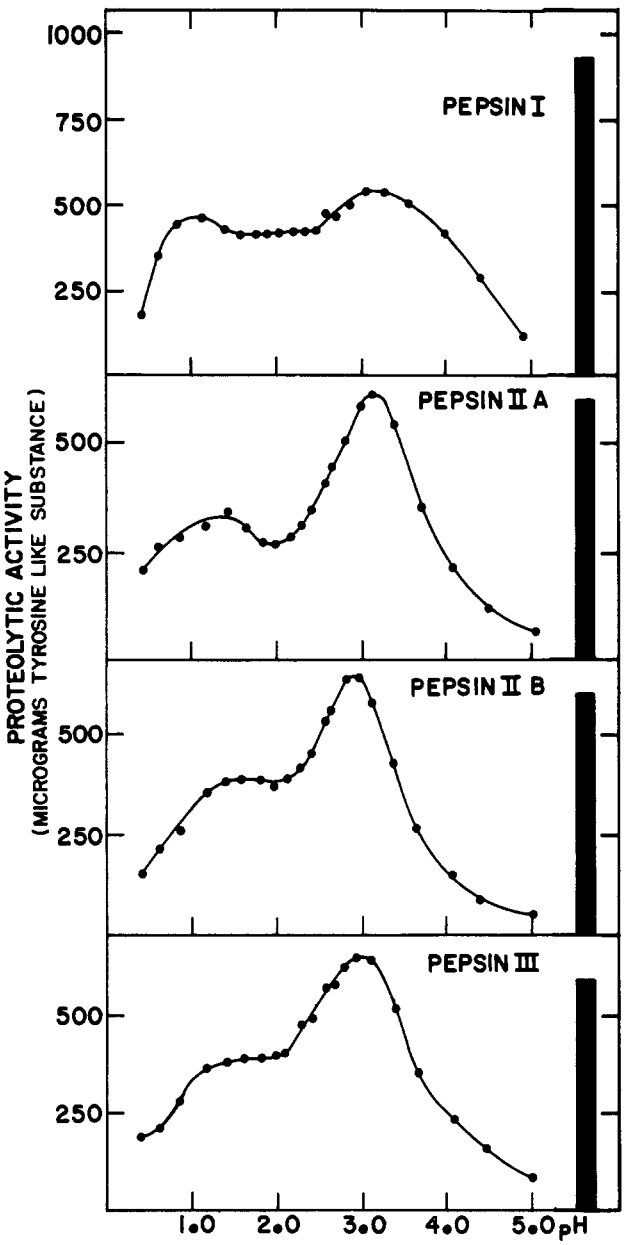

FIG. 8.—Proteolytic activity–versus–*p*H curves for proteolysis of BSA by human pepsins at 37°. Incubation mixtures: 4 ml 0.5% BSA + 1 ml HCl + 1 ml pepsin solution. Vertical bar denotes extent of proteolysis of bovine hemoglobin at *p*H 2.4–2.5 by a 1:2 dilution of the same pepsin solution.

Proteolytic activity of a 1:2 dilution of the pepsin solutions employed was assayed with acid hemoglobin at *p*H 2.4–2.5 to allow comparison of extent of substrate digestion; results are given for comparison as the solid bar in Figure 8. Human pepsins are seen to release relatively less tyrosinelike substance from BSA than from bovine hemoglobin at *p*H 2.4–2.5. However, this is most pronounced for the digestion of BSA by pepsin I.

Determination of Optimal pH of Proteolysis of BSA by Pepsin IIA at High Substrate Concentration.—This experiment was, in all details, similar to the one described above except for employment of 2.5% BSA instead of 0.5% BSA. Figure 9 depicts how high substrate concentration (2.5% BSA) and addition of sodium chloride to BSA (BSA in 0.25 M NaCl) result in broad, almost symmetrical activity-versus-*p*H curves with a "plateau" between *p*H 2.0 and 3.0.

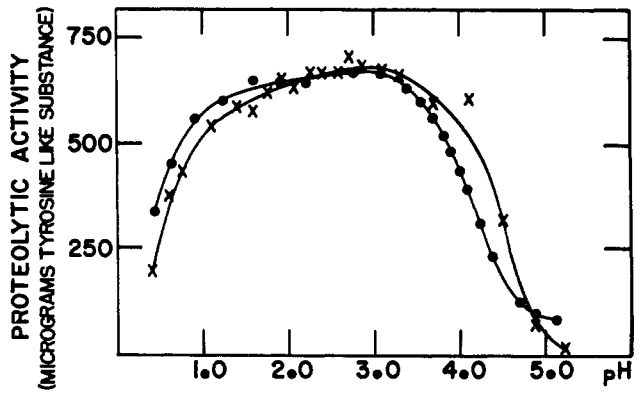

FIG. 9.—Proteolysis of BSA by human pepsin IIA at 37°. Incubation mixture: 4 ml BSA + 1 ml HCl + 1 ml pepsin solution. ●—●, 4 ml 2.5% BSA; ×—×, 4 ml 0.5% BSA in 0.25 M NaCl.

Determination of Optimal pH of Proteolysis of BSA in Sodium Chloride Solution by Pepsin IIA.—One-half per cent BSA dissolved in 0.25 M NaCl was employed as substrate. Otherwise the experiment was similar to the foregoing. Results are seen in Figure 9.

Comparison of Milk-clotting Activity at pH 4.9 with Proteolysis of Bovine Hemoglobin at pH 2.4–2.5.—A series of dilutions of each pepsin solution was assayed for proteolytic activity with acid hemoglobin as substrate, as described, and with milk as substrate to afford comparison of proteolysis over a range of pepsin concentrations. Assay for milk-clotting activity at 37° was performed as follows: Pepsin solution (0.1 ml) was pipetted into 2 ml 0.1 M acetate buffer, *p*H 4.9, and the mixture was allowed to equilibrate at 37°. The reaction was started by addition of 0.5 ml of a milk–buffer–calcium chloride mixture (5 parts fresh homogenized milk: 5 parts 0.1 M acetate buffer, *p*H 4.9: 1 part 0.1 M calcium chloride) which had been equilibrated at 37°. The *p*H of the final reaction mixture was 4.9. Clotting time expressed in minutes is the time elapsed between addition of milk and appearance of grossly visible aggregates of protein.

Figure 10 compares milk-clotting activity at *p*H 4.9 with proteolysis of acid hemoglobin at *p*H 2.4–2.5 at various pepsin concentrations. Proteolytic activity with acid hemoglobin as substrate (in micrograms tyrosinelike substance) is plotted versus milk-clotting time in minutes.

There is *no* linear relationship between amount of tyrosinelike substance released and milk-clotting time over the range of pepsin concentrations tested. The curves, however, show that pepsin solutions releasing a given amount of tyrosinelike substance from acid hemoglobin effect milk clotting at different time intervals; relative milk-clotting activity is greatest for pepsin IIB, least for pepsin IIA, while pepsins I and III have intermediate milk-clotting activity of about the same magnitude.

DISCUSSION

The present study compares properties of four closely related but different human pepsins obtained from a single stomach specimen. Extensive investigations in which pepsins in both crude and crystalline forms were employed have been reviewed recently (Bovey and Yanari, 1960). However, most data have been obtained for swine pepsin and there are only two reports concerning relatively pure human pepsin preparations (Tang *et al.*, 1959; Seijffers *et al.*, 1963a). In all reports excepting the last, the material used as a source of enzyme was a mixture from many animals or patients.

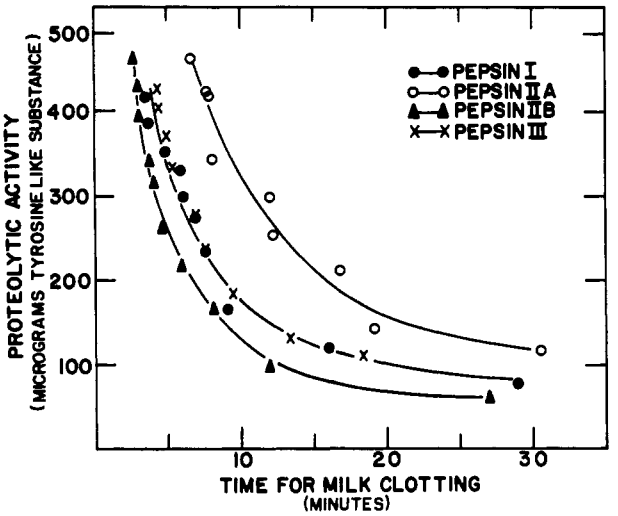

FIG. 10.—Relative substrate specificity. Proteolytic activity of human pepsins estimated with bovine hemoglobin (at *p*H 2.4–2.5) as substrate is plotted versus proteolytic activity estimated from time of milk clotting (at *p*H 4.9).

The development of an isolation method for human pepsins from fundic mucosa (Seijffers *et al.*, 1963b) has made possible the study of four different, and by chromatographic criteria, pure human pepsins. Present lack of methods for isolation of these pepsins on a large scale has limited the scope of this investigation to study of properties which could be evaluated by sensitive assays for enzymatic activity. Information pertaining to amino acid composition and structure of human pepsins awaits isolation on a larger scale.

Although not able to reveal in detail the complex progress of protein digestion, the method employed for following proteolysis of hemoglobin affords comparative results. It is to be emphasized that results obtained with this method were in the linear or almost linear range (Fig. 1). It is possible that the slight deviation from linearity observed for pepsin I is ascribable to its lability at acid *p*H (cf. Figs. 6 and 7).

Reports mentioning the existence of two *p*H optima for the digestion of proteins by human gastric juice have repeatedly appeared in the literature and have been interpreted as evidence for existence of two pepsins in human gastric juice (Buchs, 1954; Milhaud and Epiney, 1951). At times, investigators have failed to find evidence for more than one *p*H optimum (Christensen, 1955; Masch and Huchting, 1955) and the double-peaked *p*H-optimum curve has been called an artifact (Tolckmitt, 1954). Taylor (1959) has recently confirmed that human gastric juice and gastric mucosal extracts digest a number of proteins with the exception of egg albumin with two *p*H optima. However, his interpretation differs from previous workers in the postulation of two separate pyloric and fundic pepsins each digesting proteins with two *p*H optima. The study of human pepsins isolated from known anatomical portions of a single stomach and purified by chromatography has led to the following conclusions regarding the existing controversy. The notion that an activity-versus-*p*H curve with two optima is in itself indicative of the presence of two pepsins is not supported by our data. For pepsins IIA, IIB, and III the finding of two *p*H optima is, among other factors, a function of protein substrate; this may hold for pepsin I as well but is not evident from this study which has been restricted to BSA and bovine hemoglobin. Furthermore, the same protein can be digested with one or two *p*H

optima depending on substrate concentration and ionic composition of incubation mixtures. This is demonstrated for pepsin IIA acting on BSA, and has been described in more detail for pepsin III (Seijffers *et al.*, 1964a).

Previous investigations of activity versus *p*H with human pepsin mixtures have been carried out under widely different experimental conditions; this may partly explain the lack of uniformity in results, and renders comparison difficult. Nevertheless, it is of interest that Taylor's (1959) work with pepsin mixtures corroborates our finding of two *p*H optima as a function of protein substrate. The activity-versus-*p*H curve for a relatively pure human pepsin preparation acting on bovine hemoglobin has been reported (Tang *et al.*, 1959). The "gastricsin" of these authors is probably identical with what we have called pepsin I (*vide infra*); yet the activity-versus-*p*H curve of gastricsin in their report differs from the *p*H optimum curve for pepsin I described. This difference may be ascribable to differences in technique employed.

Unfortunately, hydrolysis of synthetic substrates (Baker, 1951; Fruton and Bergmann, 1939) requires far higher pepsin concentrations than have been available to us from single-stomach fractionations. Differences between the action of different human pepsins on the same protein substrate on the one hand, and differences between action of the same human pepsins on different proteins are therefore difficult to interpret at present.

The denaturation and inactivation of porcine pepsin at near neutral *p*H has been extensively studied under a variety of experimental conditions (Bovey and Yanari, 1960). This process, which is accompanied by loss of protons and unfolding of pepsin molecules, has been shown to be second order at higher pepsin concentration and lower temperatures, whereas at higher temperature (25°) and lower concentrations the reaction is first order (Bovey and Yanari, 1960). It is noteworthy that the first-order kinetics observed in our experiments with human pepsins were obtained at 25° and at low (judged by proteolytic activity) pepsin concentrations. The different rates of inactivation of these pepsins presumably reflect structural differences among them.

Changes in pepsin molecules resulting from exposure to heat at *p*H 5.4 may be partly or wholly identical to those resulting from contact with weak alkali at 25° (cf. Figs. 4 and 5). Such a unitarian concept would fail to explain why pepsin I is not more resistant than the other pepsins to heat at *p*H 5.4, unless this is related to differences in ionic composition of alkali-inactivation mixtures and heat-inactivation mixtures (see methods).

Although porcine pepsin has been reported (Green and Neurath, 1954) maximally stable at *p*H 5–5.5, this is certainly not the case for all human pepsins. Comparison of data (*vide supra*) shows clearly that with the exception of pepsin I, the human pepsins are more stable at *p*H 2.4 than at *p*H 5.4 under the experimental conditions used. Loss of activity of porcine pepsin at low *p*H has been shown to follow first-order kinetics and is accompanied by loss of protein, without perceptible formation of denatured protein. It is probable that denatured protein is formed as an intermediate step in the reaction but is hydrolyzed so rapidly that its presence cannot be detected (Northrop, 1930). In the light of the unusually low pepsin concentrations employed in our experiments (as judged by proteolytic activity), it will be of interest to observe whether autodigestion will also account for the apparent first-order inactivation of human pepsins at low *p*H.

The protective effect of bovine hemoglobin on heat-inactivation of human pepsins (with the exception of pepsin I) at *p*H 2.4 (cf. Figs. 6 and 7) is probably due to a specific stabilizing effect of substrate on enzyme (Burton, 1951; Dixon and Webb, 1958), and under the specific experimental conditions is not exerted on all pepsins to the same extent.

A comparison of protein-substrate specificity of pepsins can have no absolute value unless actual enzyme concentrations are known. Furthermore, assay of protein proteolysis with any single protein substrate reveals only the overall results of proteolysis. Within the restrictions of these limitations comparison of overall results of proteolysis of milk (expressed as clotting time in minutes) and BSA (expressed in micrograms tyrosinelike substance released) relative to proteolysis of bovine hemoglobin (expressed in micrograms tyrosinelike substance released) reveals a certain degree of relative substrate specificity (cf. Figs. 2, 9, and 10). Employment of synthetic substrates or of proteins of known primary structure such as the β chain of oxidized insulin may ultimately relate protein-substrate specificity of different pepsins to preferential hydrolysis of specific peptide chains.

The fractionation of acidified human pepsinogens on DEAE-cellulose has shown that human pepsinogen II yields two pepsin fractions, i.e., pepsins IIA and IIB. Pepsin IIA has a chromatographic mobility intermediate between pepsin I (obtained from pepsinogen I) and pepsin III (obtained from pepsinogen III). Pepsin IIB has the same chromatographic mobility as pepsin III; despite this we have hypothesized that pepsins IIB and III were different pepsins (Seijffers *et al.*, 1963c). The differences described in *p*H optimum, in resistance to heat, to alkali, and to acid, and in relative substrate specificity of the pepsin fractions studied, substantiate this hypothesis.

Pepsins IIA, IIB, and III are formed from precursors which are most probably secreted by the chief cell. However, the precursor of pepsin I originates from cell types in duodenal, pyloric, and fundic mucosa (Seijffers *et al.*, 1963b) which at present are unknown and presumably less differentiated. It is therefore of interest to note that pepsin I differs considerably from the other human pepsins in the shape of its activity-versus-*p*H curves and in resistance to acid and alkali. It may be more than coincidental that relatively alkali-resistant pepsin I is secreted in the duodenum and pylorus by glands with an alkaline secretion. Regarded teleologically, the relative resistance to acid of pepsins produced by the chief cells may have been acquired as protection against a highly acidic environment.

Few investigations have compared properties of different pepsins obtained from the same species. Tang *et al.* (1959) have studied two pepsin fractions ("gastricsin" and "pepsin") obtained from human gastric juice. "Gastricsin"[3] has been subjected by us to chromatography on DEAE-cellulose and has a chromatographic mobility similar to pepsin I (unpublished data); "pepsin" is therefore probably a mixture of pepsins IIA, IIB, and III.

We have previously reported separation and a partial characterization of two pepsin fractions from human gastric juice (Seijffers *et al.*, 1963a). In retrospect, "peak I" referred to in that report is identical to what we now designate as pepsin I, and "peak II" is a mixture of pepsins.

From crude pooled porcine pepsin Ryle and Porter (1959) separated two minor fractions which they called parapepsins I and II, and a major fraction ("pepsin"). Although these porcine pepsins were characterized it is

[3] We are grateful to Drs. J. Tang, S. Wolf, R. Caputto, and R. Trucco for a specimen of gastricsin.

not known from which part of the stomach they originate. Study of porcine fundic mucosal extracts in analogy to our studies (Seijffers *et al.*, 1963b) may reveal the presence of a fourth porcine pepsin and could more exactly reveal the quantitative relationship of these pepsins in different parts of the stomach. At present it can only be noted that parapepsin I is relatively alkali stable and that it is eluted before the other porcine pepsins from DEAE-cellulose (Ryle and Porter, 1959); in these respects it resembles human pepsin I. As most work pertaining to characterization of pepsins has been carried out with what has been designated as "crystalline porcine pepsin," it becomes clearly important to carry out studies which will establish the identity of "crystalline porcine pepsin" and its relationship to the human pepsins described in this report.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Mrs. Elizabeth Seijffers, Miss Gilda Guarasci, and Miss Gail Wildey.

REFERENCES

Anson, M. L., and Mirsky, A. E. (1932), *J. Gen. Physiol. 16*, 59.
Baker, L. E. (1951), *J. Biol. Chem. 193*, 809.
Bovey, F. A., and Yanari, S. S. (1960), *Enzymes 4*, 63.
Buchs, S. (1954), *Enzymologia 16*, 193.
Burton, K. (1951), *Biochem. J. 48*, 458.
Christensen, K. L. (1955), *Scand. J. Clin. Lab. Invest. 7*, 225.
Dixon, M., and Webb, E. C. (1958), Enzymes, New York, Academic, p. 45.
Fruton, J. S., and Bergmann, M. (1939), *J. Biol. Chem. 127*, 627.
Green, N. M., and Neurath, H. (1954), *Proteins 2B*, 1086.
Masch, L., and Huchting, I. (1955), *Z. Physiol. Chem. 301*, 49.
Milhaud, G., and Epiney, J. (1951), *Gastroenterologia 77*, 193.
Miller, L. L., Segal, H. L., and Plumb, E. J. (1957), *Gastroenterology 33*, 556.
Northrop, J. H. (1930), *J. Gen. Physiol. 13*, 789.
Ryle, A. P., and Porter, R. R. (1959), *Biochem. J. 73*, 75.
Seijffers, M. J., Miller, L. L., and Segal, H. L. (1963a), *J. Am. Med. Assoc. 183*, 998.
Seijffers, M. J., Miller, L. L., and Segal, H. L. (1964a), *Biochemistry 3*, 1.
Seijffers, M. J., Miller, L. L., and Segal, H. L. (1964b), *Biochemistry 3*, 5.
Seijffers, M. J., Segal, H. L., and Miller, L. L. (1963b), *Am. J. Physiol. 205*, 1099.
Seijffers, M. J., Segal, H. L., and Miller, L. L. (1963c), *Am. J. Physiol. 205*, 1106.
Tang, J., Wolf, S., Caputto, R., and Trucco, R. E. (1959), *J. Biol. Chem. 234*, 1174.
Taylor, W. H. (1959), *Biochem. J. 71*, 73.
Tolckmitt, W. (1954), *Biochem. Z. 325*, 389.

Structural Studies of Ribonuclease. XIV. Tryptic Hydrolysis of Ribonuclease in Propyl Alcohol Solution*

TATSUO OOI† AND HAROLD A. SCHERAGA

From the Department of Chemistry, Cornell University, Ithaca, N. Y.

Received April 16, 1964

The unfolding of ribonuclease occurs at a lower temperature in aqueous *n*-propyl alcohol solution than in water, presumably because of the effect of the alcohol on hydrophobically bonded regions of the ribonuclease molecule. Tryptic digestion of ribonuclease in 25% propanol at 35° was employed to determine the region of the ribonuclease molecule which unfolds in aqueous alcohol. The digestion products were purified by IRC-50 chromatography. Two major components (*a* and *b*) were obtained. On the basis of amino acid composition and N- and C-terminal-group data, component *a* was found to be identical with component 1, a species obtained by digestion of component IV by trypsin in water at room temperature (Ooi and Scheraga, 1964a); component *b* is unreacted ribonuclease plus a small amount of component 5, obtained along with component 1 from component IV. Therefore the same regions of the ribonuclease molecule (between Lys 31 and Lys 37) are involved in the thermal transitions in 25% propanol at 35° and in water at 60°, respectively. Both transitions presumably involve the disruption of the same hydrophobically bonded regions of ribonuclease.

Even though trypsin and chymotrypsin do not digest native ribonuclease at room temperature, they do attack it at elevated temperatures, where the substrate undergoes a thermal transition (Rupley and Scheraga, 1963; Ooi *et al.*, 1963; Ooi and Scheraga, 1964a,b). In fact, it has been possible to identify the regions of the ribonuclease molecule which are involved in the thermal transition by determining the sites of initial proteolysis by trypsin and chymotrypsin, respectively.

The thermal-transition temperature of ribonuclease is lowered by the addition of alcohols at neutral *p*H (Schrier and Scheraga, 1962). This result has been attributed to the breaking of hydrophobic bonds in ribonuclease. It is thus conceivable that the parts of the chain which are exposed upon heating in the presence of alcohol might be different from those exposed when the heating is carried out in the absence of alcohol. If this were the case, then differences might be found in the sites of initial proteolysis by trypsin. Therefore experiments were carried out to determine whether trypsin is active in *n*-propyl alcohol and, if so, to identify the regions of the ribonuclease molecule which are attacked by trypsin in the presence of alcohol.

* This work was supported by a research grant (AI-01473) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, U. S. Public Health Service, and by a research grant (GB-75) from the National Science Foundation.

† On leave from the Department of Physics, Nagoya University, Nagoya, Japan, 1961–63.