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AN ENZYME SYSTEM IN THE GASTRIC SECRETION CAPABLE OF REDUCING THE VISCOSITY OF NATIVE SOLUBLE CALF-SKIN COLLAGEN

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SUMMARY

Gastric juice from various animals contained an enzyme system capable of reducing the viscosity of a solution of native calf-skin collagen. These enzymes were confined in their action to the end-chains of collagen and could not be classified as collagenases. The end-chains contributed approx. 30% of the initial solution viscosity of collagen. Commercial preparations of pepsin (EC 3.4.4.1) differed in their ability to reduce the viscosity of collagen. Chromatographically homogeneous pepsin and three chromatographic subfractions (pepsins B, C and D) were demonstrated to have different actions on soluble collagen as indicated by viscosity changes. The individual pepsin enzymes had characteristic affinities for collagen and probably had different substrate specificities. The different actions of commercial pepsins on collagen could be accounted for by the presence of pepsin plus one or more of the more active subfractions. It was suggested that one sample of commercial crystalline pepsin and also human gastric juice contained a pepsin-like enzyme, other than any of the subfractions at present isolated, which was very much more active than any purified pepsin.

INTRODUCTION

Collagen can be denatured by heating[1] or chemical treatment[2] to yield a gelatin which is readily hydrolysed by proteolytic enzymes. Native collagen, however, is almost completely resistant to proteolytic digestion by enzymes other than collagenases (i.a. EC 3.4.4.19)[3]. Native collagen is considered to be made up from highly organised regions rich in non-polar amino acids and imino acids interspersed between regions containing polar amino acids[4]. These two types of structures constitute the main part of the native collagen molecule and they are often described as the crystalline and amorphous regions respectively. HODGE *et al.*[5] demonstrated the presence of random coil peptide chains attached to the ends of the collagen monomer or tropocollagen molecule. These authors claimed that the end-chains could be hydrolysed by trypsin (EC 3.4.4.4), pepsin (EC 3.4.4.1) and chymotrypsin (EC 3.4.4.5) with the result that the ability to undergo end-to-end polymerisation was destroyed in the modified collagen. This claim has been confirmed in the case of pepsin digestion by RUBIN *et al.*[6]. Pepsin removed a small peptide appendage, approx. 200 Å long, from the

end of the tropocollagen molecule and this resulted in a reduction of the intrinsic viscosity of the protein solution by approx. 40% (see ref. 7).

A true collagenase might be defined as an enzyme capable of hydrolysing peptide bonds within the crystalline regions of native collagen with the release of peptides rich in proline and hydroxyproline. The chemical structure of the crystalline regions is ideally suited to the substrate specificity of bacterial collagenases[3,8–11]. Enzymes which were capable of a limiting type of hydrolysis, confined to the random coil end-chains of native collagen, would fail to qualify as true collagenases as defined above.

The original object of this study was to determine whether carnivorous animals possessed a gastric enzyme system capable of acting as a true collagenase. It was found that stomach juice enzymes from a variety of animals were capable of a strictly limited attack on the end-chains of collagen. The present work describes these enzyme systems and indicates a method which employs the viscosity changes in a solution of native collagen to differentiate the actions of four subfractions of pepsin and the pepsin-like enzyme of the stomach.

MATERIALS

Acetic acid soluble collagen was prepared from calf-skin as previously described[12]. Gastric secretion was withdrawn from fasted humans and cats by stomach tube. In the case of human gastric juice which contained mucin, the protein was freed from the mucin by addition of a few drops of 3 N acetic acid which caused the mucin to aggregate, the protein solution was then filtered and used for enzyme studies. Pooled gastric juice obtained from hospital patients had previously been stored at —20° and on thawing the mucin was readily removed by filtration. An attempt was made to concentrate the enzymes of the gastric juice from a pooled hospital sample as follows. Solid ammonium sulphate was added to the filtered gastric juice and the mixture stirred until the solution was approx. 80% saturated with respect to ammonium sulphate. A sample of the precipitated protein was collected by centrifugation and after exhaustive dialysis was found to contain an active enzyme system.

Rabbits, rats, hamsters and guinea-pigs were starved for 48 h prior to killing, the stomachs were tied off, removed, and the stomach contents collected. Centrifugation and filtration of the supernatant fractions from these stomach contents yielded the enzyme solutions which were examined in this work.

Porcine pepsin preparations were obtained from the following sources:
Crude pepsin, B.D.H. 1:2500 powder No. 394770. Crystalline pepsin, Armour No. 22306. Twice crystallised pepsin, Mann No. C 2253.

Dr. A. P. RYLE of Edinburgh University very kindly provided a sample of pepsin from which pepsins B and C had seem separated by chromatography and which behaved as a single peak on rechromatography. Since this material was the major chromatographic fraction obtained from crude porcine pepsin it is here referred to as pepsin in contrast to the cruder commercial pepsins. The name pepsin A has previously been employed by HERRIOTT *et al.*[14] and therefore cannot now be used in connection with RYLE's[15,16] main chromatographic component. Minor chromatographic components isolated from crude pepsin which had enzymic activity similar to pepsin are referred to as pepsins B, C, and D. Pepsins B and C were previously described by RYLE AND PORTER[13] as parapepsins I and II respectively. Pepsin D has recently been isolated by LEE AND RYLE[17].

METHODS

Stock solutions of collagen were prepared in 0.1 M acetic acid with pH 3.5 and contained 1 mg collagen/ml. Collagen concentrations were determined by microkjeldahl estimation employing a value of 18.0% for the protein total nitrogen[12]. The total protein concentration of a known volume of stomach juice was determined by precipitation of the protein with an equal volume of 20%(w/v)trichloroacetic acid followed by nitrogen analysis of the precipitate. The protein concentration was then calculated on the assumption of an average total nitrogen content of 16% for the gastric juice proteins.

Viscosities were measured at 19° in an Ostwald viscometer with a flow time for water of 69 sec. The collagen solution (5 ml) was mixed in the viscometer with 0.1 ml enzyme solution and the change in viscosity measured over 1 h; extra time was allowed when necessary for the mixture to reach a limiting viscosity value.

RESULTS

The results of these investigations are presented graphically in Figs. 1–4. When a limiting viscosity value had been reached no further reduction in viscosity was

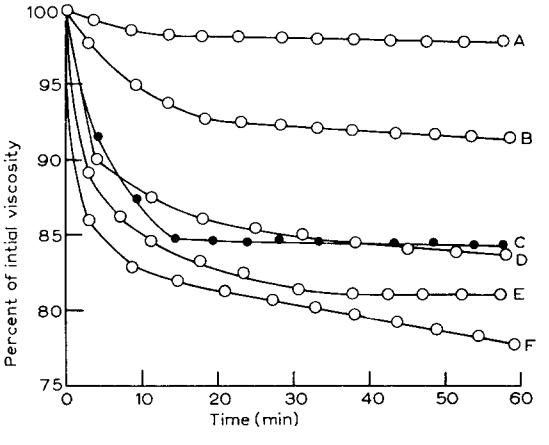

Fig. 1. Change in viscosity of soluble calf-skin collagen at 19° in the presence of animal stomach-juice enzymes. A, Hamster; B, rat and guinea pig; C, cat; D, rabbit; E, washings from guinea-pig-stomach lining; F, rabbit juice obtained after storing the stomach at 4° for 30 min prior to analysis. Ordinate, $\Delta\eta$ (percentage of initial value); abscissa, time (min).

observed on mixing a second addition of the enzyme solution to the system and this was taken as evidence that the enzyme reaction had reached completion. In other experiments with commercial crystalline pepsin (Mann with $E:S$ ratio of 1:1) no greater reduction in viscosity was produced than in the experiments employing an $E:S$ ratio of 1:10. These observations indicated that the experimental conditions were not causing inhibition of the enzyme systems before the enzyme reaction had reached completion.

DISCUSSION

The change in the viscosity of soluble collagen brought about by various animal gastric juice preparations are presented in Fig. 1. Hamster gastric juice produced the least change although there was a definite indication of enzymic activity (Curve A). Rat, guinea-pig, rabbit and cat juices produced more marked changes in the viscosity of soluble collagen. These results are not strictly comparable since the concentration of active enzymes in these gastric juices was not known. The limiting viscosity values are a better guide to the degree of digestion to which each of these enzyme systems was capable of degrading the native tropocollagen molecule.

It was observed that the activity of the gastric juice appeared to increase if the stomach was stored at 4° for 30 min before the viscosity study was carried out (*e.g.* Fig. 1, F). This increased activity may be due to the release of intracellular enzymes, as for example the cathepsins which have often been claimed to have collagenolytic activity[18]. Washings from the stomach lining of a guinea-pig were more active than the fresh stomach juice of the same animal (Fig. 1, Curves B and E). In this instance the tissue was rubbed gently between the fingers with a few drops of distilled water in order to collect the washings.

Human gastric-juice preparations were examined and the changes in collagen viscosity observed are presented in Fig. 2. Total protein substrate ratios (P:S) were calculated from protein nitrogen estimations performed on the gastric-juice samples. The actual enzyme substrate ratios could not be determined. Curve G of Fig. 2 indicates the activity of a protein concentrate obtained from pooled stomach juice by ammonium sulphate precipitation followed by dialysis of the protein before examining its action on the collagen solution. This material was capable of reducing the viscosity of soluble collagen to 68.5% of its initial value as did the most active preparations of fresh gastric juice (Curves E and F in Fig. 2).

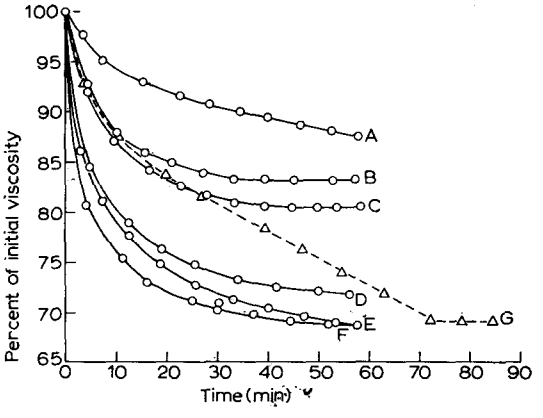

Fig. 2. Change in viscosity of soluble calf-skin collagen at 19° in the presence of human gastric juice. A, sample G.S. treated with acetic acid to precipitate mucin (P:S = 1:260); B, sample J. McE. treated as A (P:S = 1:170); C, pooled hospital sample I, mucin removed after thawing from −20° (P:S = 1:140); D, sample J. C. treated as A; E, sample J. B. containing no mucin; F, sample J. K. containing no mucin (P:S = 1:150); G, protein fraction precipitated by 80% saturated ammonium sulphate from pooled hospital sample II. Ordinate, $\Delta\eta$ (percentage of initial value); abscissa, time (min).

The presence of gastric enzymes capable of reducing the viscosity of native soluble collagen at pH 3.5 suggested that pepsin might possibly be involved. It was therefore of interest to examine the activity of commercial pepsin preparations on a comparable weight basis by employing an $E:S$ ratio of 1:10. Three commercial pepsin samples were examined and the results are presented in Fig. 3. In the case of crude pepsin from B.D.H. a weight ratio of 1:10 was used and this was equivalent to a total protein substrate ratio of 1:185. These commercial pepsin preparations differed markedly in their ability to reduce the viscosity of soluble collagen. In each case the viscosity fell to a constant limiting value; the lowest value was reached by the Mann preparation (*viz.* 69%) and this is again significant in that the lowest value reached

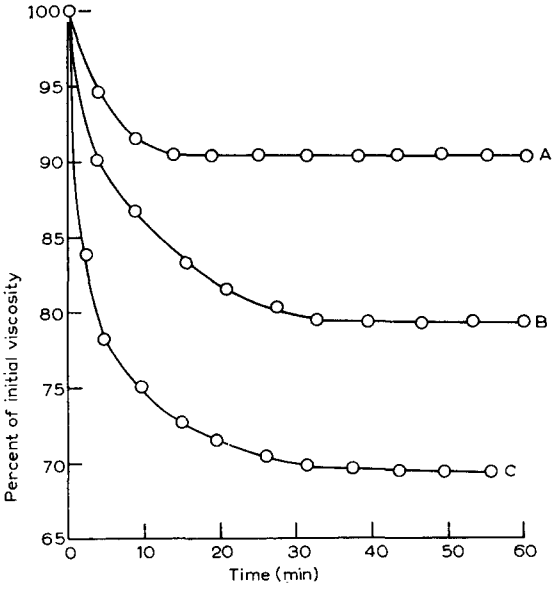

Fig. 3. Change in viscosity of soluble calf-skin collagen at 19° in the presence of commercial pepsins. A, Crystalline pepsin (Armour No. 22306) ($E:S$ = 1:10) B, crude pepsin (B.D.H. 1:2500 powder No. 394770) weight-substrate ratio 1:10 ($P:S$ = 1:185); C, twice crystallised pepsin (Mann No. C 2253) ($E:S$ = 1:10). Ordinate, $\Delta\eta$ (percentage of initial value); abscissa, time (min).

in the stomach-juice preparations was 68.5%. These observations indicated that various commercial pepsin preparations might contain at least three distinct enzymes with characteristic specificities and that individual enzymes of this system would be capable of reducing the collagen viscosity to 90.5%, 79.0% and 69.5% of the initial value. This theory was confirmed by co-operation with Dr. RYLE who very kindly provided samples of four pepsin fractions obtained by chromatographic fractionation of crude pepsin or neutral extracts of pig mucosa.

The activities of the four pepsin sub-fractions were examined on soluble collagen employing an $E:S$ ratio of 1:10 and the results are presented in Fig. 4. In the presence of each pepsin sub-fraction collagen exhibited a characteristic viscosity *versus* time curve and limiting viscosity value. In three of these pepsins the limiting viscosity values were almost identical with those observed in the commercial pepsins. It is

probable that commercial pepsin from Armour contained only the major chromatographic component here referred to as pepsin, crude pepsin from B.D.H. contained pepsin contaminated with pepsin C and the Mann preparation contained pepsin as well as pepsin D with the possibility of contaminated pepsin C as well. It would also appear that human gastric juice contained an enzyme system equivalent to pepsin, plus pepsins C and D (see Fig. 2, Curves F and G) and that acetic acid precipitation of the mucin in human gastric juice (Fig. 2, Curves A and B) caused a loss in pepsin D activity. In this discussion the influence of pepsin B has been ignored since its activity is closely similar to the major component, pepsin.

An *ad hoc* mixture was prepared containing pepsin plus pepsin B and C in the weight ratios 8:1:1 respectively and this mixture reacted with soluble collagen using a total E:S ratio of 1:10 (Curve E of Fig. 4). The viscosity *versus* time curve initially

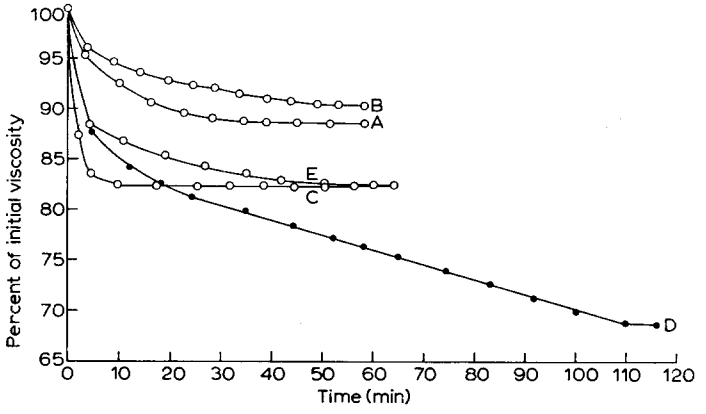

Fig. 4. Change in viscosity of soluble calf-skin collagen at 19° in the presence of pepsin subfractions obtained by chromatography and provided by Dr. RYLE. In each experiment E:S was 1:10. A, Main chromatographic component referred to as pepsin; B, sub-component pepsin B; C, sub-component pepsin C; D, sub-component pepsin D; E, mixture of pepsin, pepsin B and pepsin C in the ratio 8:1:1 respectively employing a total E:S ratio of 1:10. Ordinate, $\Delta\eta$ (percentage of initial value); abscissa, time (min).

falls rapidly and then flattens out more slowly in the second stage of the reaction to reach a limiting viscosity value of 82.5%, a value identical to that reached by pepsin C alone (Fig. 4, Curve C). The initial steep fall in Curve E reflects the combined actions of the major constituent, pepsin, of the mixture and the more reactive though less concentrated constituent, pepsin C. This first stage rapidly brings the viscosity down to 88% in a manner similar to that which is produced by pepsin (see Curve A of Fig. 4). The slower second part of Curve E is mainly a reflection of the action of pepsin C and this part of the curve has a time base roughly ten-fold that observed in Curve C for pepsin C containing a ten-fold concentration of the enzyme. In the theoretical analysis of Curve E pepsin B has been ignored since it is the least active pepsin preparation and is present in an effective E:S ratio of 1:100.

The very high value of laevorotation[19] associated with the "collagen fold" is thought to be due to the presence of poly-proline-like helices[20] found in the imino acid rich crystalline regions of native tropocollagen. It was observed that commercial

pepsin (Mann E:S ratio 1:10) in the presence of soluble collagen produced virtually no change in the optical rotation after 1 h during which time the viscosity had been reduced to 69% of the initial value. The non-protein nitrogen obtained by acetone precipitation of the enzyme reaction mixture was hydrolysed and found to contain no more proline and hydroxyproline than was released from a control experiment in which the enzyme was added to the collagen after acetone precipitation of the protein. It is concluded that neither the gastric secretion enzymes nor any of the pepsin preparations were capable of attacking the crystalline regions of native collagen, unlike bacterial collagenases which cause a reduction in both viscosity and laevorotation as well as releasing peptides rich in imino acids. The gastric secretion enzymes and purified pepsins reduce the viscosity of soluble collagen by a limited attack on the end-chains in a manner similar to that described by HODGE *et al.*[5]. NISHIHARA[7] has recently reported that pepsin can hydrolyse this type of end-chain. It is worth noting that the rate of viscosity fall produced by pepsin D alone does not account for the much more rapid fall in viscosity produced by gastric juice at a much reduced enzyme strength (see Fig. 2, Curves E and F) although the limiting viscosity was the same. This might suggest the presence of another pepsin-like enzyme in the stomach juice capable of reducing the collagen viscosity to 69% of the initial value. The commercial crystalline pepsin supplied by Mann exhibited a similarly rapid action and this would again lend strength to the suggestion of a pepsin sub-fraction other than those already isolated, which may be present in both human-gastric secretion and also the pepsin supplied by Mann.

It is suggested that the contribution of the end-chain peptides to the solution viscosity of native collagen amounts to about 30–32% in the conditions of these experiments. This value would seem rather high in view of the relatively small size of the end-chains compared to the overall size of the tropocollagen molecule[7]. Dimers and trimers[5] of tropocollagen are present in solution and the digestion of the end-chains involved in end-to-end polymerisation might well cause a considerable reduction in solution viscosity. NISHIHARA[7] noted a 40% reduction in viscosity of collagen after pepsin digestion and this reduction is in fair agreement with the work reported here. Evidence has now been provided for the destruction of inter-chain cross-linkages in collagen by pepsin digestion[6].

The viscosity *versus* time curve for each of RYLE's enzyme fractions is characteristic for that fraction and this observation suggests that each fraction has its own specificity requirements and this might well account for the rather wide range of peptide bonds hydrolysed by commercial preparations of pepsin[21]. TAYLOR[22] has drawn attention to the two optimal pH values observed with pepsin and has suggested the synthesis of two pepsins in different regions of the stomach to account for these observations. TANG *et al.*[23] have isolated an enzyme similar to pepsin which they have described as gastricsin. The subfractionation of pepsinogen into four distinct zymogens each related to a distinct enzyme carried out by RYLE *et al.*[13,15,16] and the present work supporting the claim of at least four pepsin enzymes may help to simplify the confusion which exists at present in connection with the peptic enzymes of gastric secretion. The high viscosity of soluble collagen and the limited number of sites available for pepsin attack make this protein a useful substrate on which to characterise these enzymes.

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NOTE ADDED IN PROOF

In a personal communication Dr. RYLE demonstrated that the chromatographically pure major pepsin component (here referred to as pepsin) caused a greater reduction in the viscosity of soluble collagen when used at higher enzyme concentrations. This observation has now been confirmed in this laboratory. Pepsin reacted with soluble collagen at $E:S$ of 1:5 and 1:2 caused the viscosity to fall to a final value of 77% and 72% respectively of the initial value.

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