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AN EXTRACTABLE COLLAGENASE FROM CRUSTACEAN HEPATOPANCREAS

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SUMMARY

An enzyme capable of degrading native collagen under physiologic conditions has been extracted from the hepatopancreas of a crustacean, *Uca pugilator*. The collagenase acts on native collagen fibrils and on collagen in solution. At 25° the enzyme is capable of producing a marked reduction in the specific viscosity of collagen with no loss of optical rotation, indicating the ability of this enzyme to cleave the native collagen helix without producing denaturation.

Collagenolytic activity, as investigated by disc electrophoresis, results in the production of numerous new components below the original α band. Three fragments, 75% (TC^{A}_{75}), 70% (TC^{A}_{70}) and 67% (TC^{A}_{67}) the molecular length from the "A" or N-terminal end of the molecule, have been identified in electron micrographs of segment-long-spacing crystallites prepared from enzyme–collagen reaction mixtures.

Crude collagenase preparations from this digestive organ also contain significant chymotrypsin- and trypsin-like activities. The cleavage by hepatopancreas extracts of peptide bonds in the nonhelical, N-terminal region of collagen, at or near the intramolecular cross-link, may be due to the action of chymotrypsin on collagen. Chymotrypsin, however, is unable to degrade α chains further at 25°. Trypsin has little effect on the viscosity of collagen in solution at concentrations equivalent to those present in hepatopancreas extracts. At these concentrations trypsin is unable to reduce the content of cross-linked components in collagen or catalyze cleavages in the native helix. In addition, the ability of hepatopancreas extracts, but not chymotrypsin or trypsin, to degrade collagen in fibrillar form further indicates the presence of a true collagenase in crustacean hepatopancreas.

INTRODUCTION

Many crustaceans are predacious scavengers that feed on animal tissues frequently containing collagen as a constituent protein. The major organ involved in the digestion of ingested protein in these organisms is the hepatopancreas[1,2]. A collagen-

olytic enzyme capable of degrading native collagen at physiologic pH and temperature has recently been demonstrated in the hepatopancreas of the crab[3]. The enzyme was found to be extractable from fresh tissue, while extracts from other animal tissues have consistently failed to yield an enzyme that will attack native collagen under physiologic conditions[4].

Collagenases have now been isolated and partially characterized from tadpole tailfin[5,6], rat uterus[7], rheumatoid synovial tissue[8], and human skin[9]. These enzymes can only be isolated from the medium of cultured tissues, although a collagenase having properties similar to the enzymes obtained from these sources, has recently been extracted directly from the granule fraction of human granulocytic leukocytes[10].

The present study demonstrates the presence of a collagenase from the hepatopancreas of the "fiddler crab", *Uca pugilator*, and partially characterizes its mechanism of attack on the collagen molecule.

MATERIALS AND METHODS

Isolation of collagenase from hepatopancreas

Hepatopancreas obtained from 75–100 freshly collected *Uca pugilator* (mixed sexes) was used as the source of enzyme. The total wet weight of hepatopancreas after removal was approx. 8–10 g. The glands were homogenized by hand in an all-glass homogenizer with 3 vol. of cold 0.05 M Tris–HCl (pH 7.4) containing 5 mM $CaCl_2$ and were centrifuged in the cold at 50 000 $\times g$ for 30 min; the supernatant was filtered through glass wool to remove the lipid material present at the surface. The pellet was reextracted with 3 vol. of the same buffer and centrifuged; the two supernatants were combined. The combined supernatant solutions were then centrifuged at 105 000 $\times g$ and 4° for 60 min to provide a clarified enzyme extract.

Assay procedures

Collagenolytic activity was determined either viscometrically or by release of soluble radioactivity from ^{14}C-labeled reconstituted collagen fibrils[6,9]. Viscosity was measured in Ostwald viscometers with flow times for water ranging from 26–32 sec at 25°. Collagen was purified by the method of GROSS[11]. For the assays, stock solutions of collagen were prepared as previously described for tadpole[4,6] and human-skin collagenases[9].

Chymotrypsin-like activity in hepatopancreas extracts was assayed by the method of SCHWERT AND TAKENAKA[12]. Trypsin-like activity was measured by the method of HUMMEL[13].

Protein was determined by the method of LOWRY *et al.*[14].

Disc electrophoresis

Thermally denatured collagen in reaction mixtures was electrophoresed in polyacrylamide gels according to NAGAI *et al.*[15]. Addition of sufficient 0.1 M HCl to the reaction mixtures to reduce the pH to approx. 2 (0.03 M) was essential to prevent further enzyme action during thermal denaturation of the collagen.

Optical rotation and electron microscopy

Optical rotation was followed simultaneously with viscosity measurements in a Cary 60 spectropolarimeter at 240 mμ and 25°.

Segment-long-spacing aggregates of collagen and enzymatic products of collagen were prepared for electron microscopy according to GROSS *et al.*[16]. Grids were positively stained with uranyl acetate and examined in a Phillips EM 300 electron microscope

Comparison of hepatopancreas collagenase to chymotrypsin and trypsin

The effects of hepatopancreas collagenase, chymotrypsin and trypsin on native acid-extracted collagen were compared in experiments similar to those described by BORNSTEIN *et al.*[17]. A final weight ratio of collagen to chymotrypsin of 10:1 and of collagen to trypsin of 50:1 was used. Reactions were monitored by viscometry and aliquots were taken at intervals for acrylamide gel electrophoresis. Repeated purification of the trypsin-treated collagen prior to electrophoresis was essential to insure complete removal of the enzyme[17].

RESULTS

The collagenase extracted from crustacean hepatopancreas acts on native reconstituted collagen fibrils at pH 7.5 and 37° and activity was linear with respect

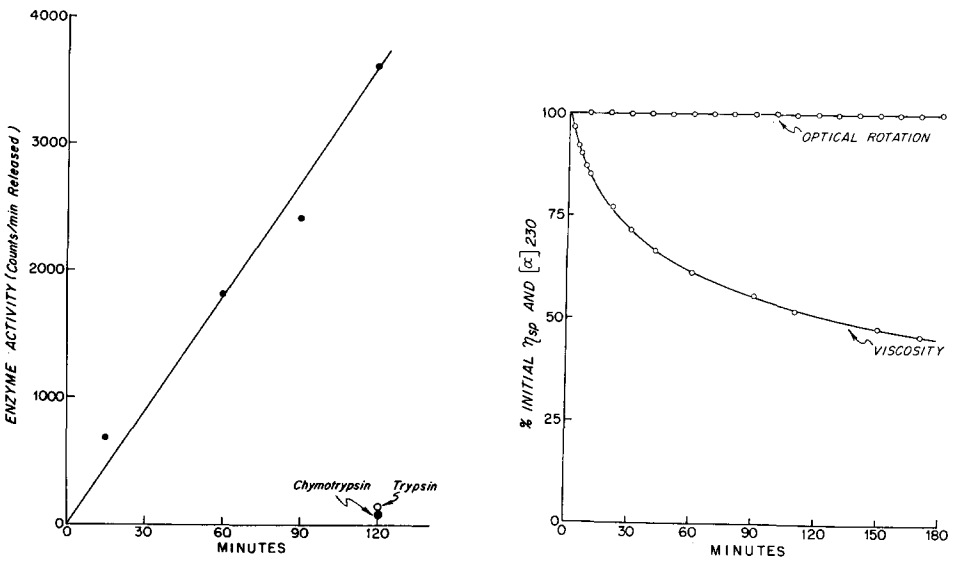


Fig. 1. Degradation of native, reconstituted collagen fibrils by 0.2 mg of hepatopancreas collagenase, measured by release of radioactivity from [^{14}C]glycine-labeled collagen gels. Trypsin and chymotrypsin controls contained 0.1 mg protein. 4460 counts/min per substrate gel.

Fig. 2. Effect of hepatopancreas collagenase on viscosity and optical rotation at 25° and pH 7.4. Starting viscosity, $\eta_{sp} = 3.0$. Control viscosity remained unchanged over the experimental time period. The optical rotation was monitored simultaneously in an aliquot of the reaction mixture. Enzyme protein concentration was 32 μg/ml.

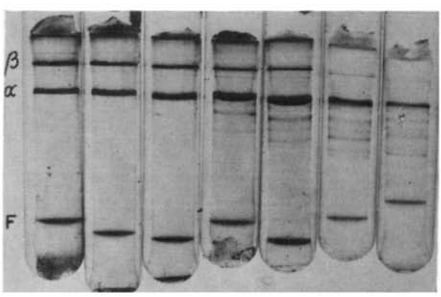
to time (Fig. 1). Dependence of activity on enzyme protein concentration, however, was less ideal, possibly because of the presence of higher concentrations of other proteolytic enzymes in crude extracts. When 1.0 mg of crude enzyme was incubated with 10 mg of intact rat-tail tendon for 12 h at 37°, over 95% of the total hydroxyproline present in the tendon collagen was released as compared to no hydroxyproline released in the absence of enzyme, demonstrating that this collagenase can degrade native tissue collagen.

Hepatopancreas collagenase is also active against collagen in solution. At pH 7.5 and 25° it is capable of reducing the initial specific viscosity of the collagen solution by 55% in 3 h at relatively low protein concentrations (Fig. 2). No accompanying change in optical rotation was noted during this period of observation. The enzyme continued to act on the collagen so that by the end of 8 h of incubation more than 80% of the initial specific viscosity was lost. Higher concentrations of enzyme caused a small but detectable loss in optical rotation at viscosity losses comparable to those seen at lower enzyme levels. This may be related to the presence of other proteases in the crude enzyme preparation that when added to the reaction mixture in increased concentrations are capable of degrading the products resulting from the action of hepatopancreas collagenase on collagen. Therefore, all experiments were performed at enzyme concentrations at which no loss of optical rotation occurred.

Exhaustive dialysis of reaction mixtures of collagen in solution and hepatopancreas collagenase, incubated at 25° until 50–80% of the initial specific viscosity was lost, yielded less than 7% dialyzable peptides.

Acrylamide gel electrophoresis

To determine the number and relative size of the collagen fragments formed, the denatured products of enzymatic attack from reaction mixtures incubated at 25°



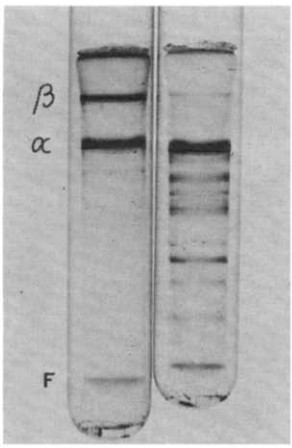


Fig. 3. Acrylamide gel electrophoresis patterns of thermally denatured enzyme–guinea pig skin collagen reaction mixture. Left to right: zero time reaction mixture, and after 10, 20, 40, 50, 60 and 70% reduction in specific viscosity. F refers to the buffer front.

Fig. 4. Disc electrophoretic pattern of thermally denatured enzyme–collagen reaction mixtures at 25°. On the left, zero time reaction mixture and on the right, after 80% reduction in specific viscosity.

were examined by acrylamide gel electrophoresis (Fig. 3). At zero time, only the α and β bands of normal collagen were present, with the γ and higher-molecular-weight aggregates remaining at the top of the running gel. At a time when the specific viscosity of the reaction was decreased by approximately 20%, little change was seen in the electrophoretic pattern. From 20–40% loss of specific viscosity striking changes in the electrophoretic pattern occurred. There was a marked decrease in the original β band and a corresponding increase in the density of the α band. Two new bands were now distinctly seen beneath the β component and new bands were also evident below the original α component. When the specific viscosity of the reaction mixture had decreased by 60–70%, the original β band had almost disappeared and there was an accentuation of the new bands below the original α component. Fig. 4 shows the electrophoretic pattern of a reaction mixture which had lost 80% of its initial specific viscosity and demonstrates clearly the presence of numerous new components beneath the original α band as well as the marked decrease in the original β component. Identical patterns were also obtained at temperatures as low as 10°.

Electron microscopy

Estimation of the size of modified fragments produced by the action of hepatopancreas collagenase was obtained by examining segment-long-spacing crystallites. Three fragments, one 75% (TC^{A}_{75}) the molecular length from the "A" or N-terminal end of the molecule (Fig. 5A), and the others approx. 70% (TC^{A}_{70}; Fig. 5B) and 67% (TC^{A}_{67}; Fig. 5C), also from the "A" end of the molecule, have so far been identified. Short segments representing the remainder of the length of the collagen molecule from the "B" end (TC^{B}) have not as yet been identified.

Comparison between hepatopancreas collagenase, chymotrypsin and trypsin

Certain proteolytic enzymes such as chymotrypsin, trypsin, pepsin and pronase are capable of releasing peptides from native collagen, without altering the helical structure[18–22]. BORNSTEIN *et al.*[17], have clearly demonstrated that chymotrypsin converts both β components and α chains to altered α chains by removing a short segment from the nonhelical N-terminal end of the chains which contain the covalent intramolecular cross-link.

Crude hepatopancreas extracts were, therefore, assayed directly for activity using specific substrates for chymotrypsin and trypsin, and found to contain about 1% by weight of chymotrypsin and approx. 33% by weight of trypsin assuming that the molecular weights of these enzymes in hepatopancreas are equivalent to those of other animal species.

Since significant levels of chymotrypsin- and trypsin-like activities are present, these enzymes were examined with respect to their ability to degrade the collagen molecule. Chymotrypsin acts on collagen in solution at 25°, reducing the initial specific viscosity by approx. 45% in 8 h. The reaction tended to plateau with an additional drop in viscosity of only 10% in the ensuing 16 h.

The acrylamide gel patterns of control collagen and the products of chymotrypsin treatment taken at varying intervals of the reaction are illustrated in Fig. 6. A progressive decrease in the β components was evident and was associated with a concomitant increase in the α band. After 24 h the β component had largely disappeared

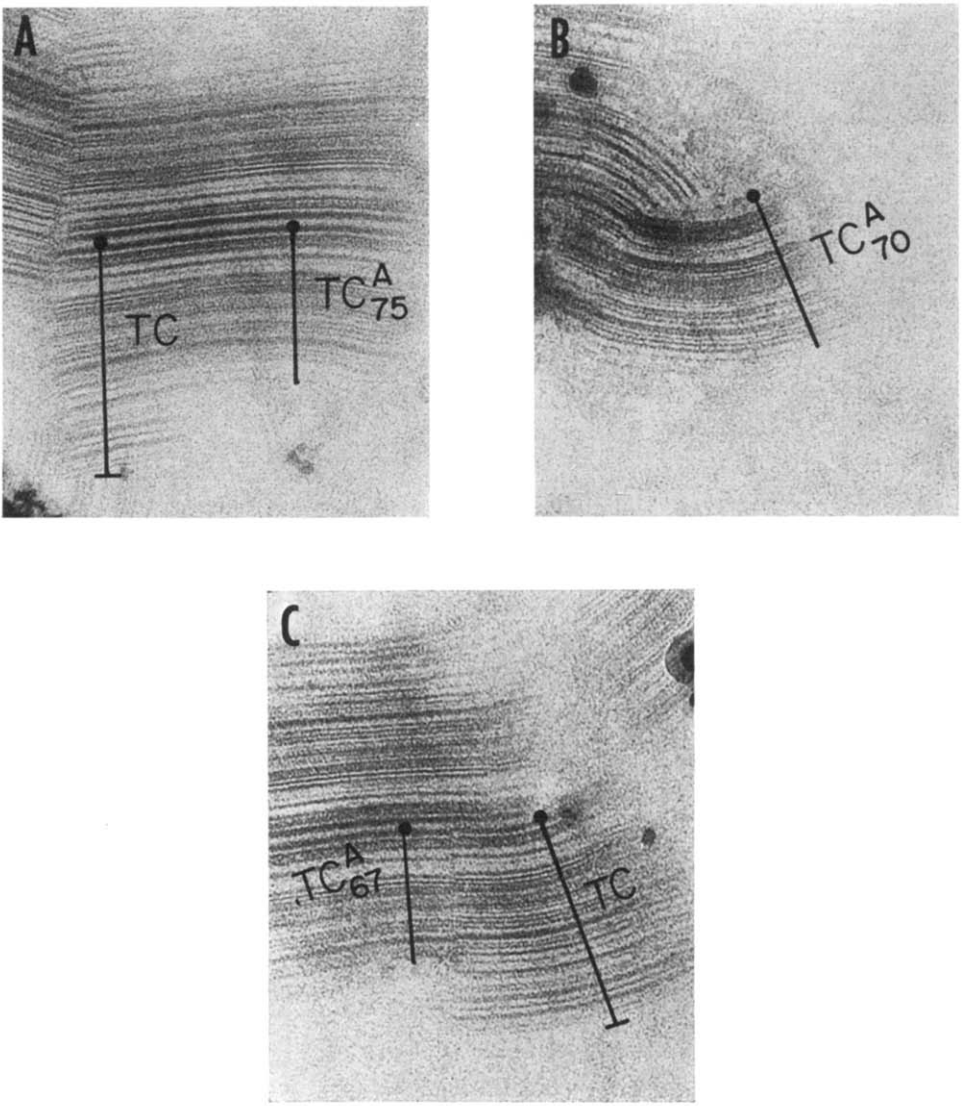


Fig. 5. Segment-long-spacing crystallites of hepatopancreas collagenase-modified collagen. A, depicts a dimer (A to A end) of two hybrid segments containing both normal length (TC) and three-quarter length (TC^{A}_{75}) segments. Magnification 97 600 ×. B, illustrates an segment-long-spacing segment that is approx. 70% normal length from the A end (TC^{A}_{70}). Magnification 97 600 ×. C, shows a normal length segment-long-spacing segment (TC) matched closely with a modified segment approx. 67% normal length (TC^{A}_{67}). The modified segment is in A to A end apposition to another normal segment. Magnification 97 600 ×.

and an intensely staining band was present in the region of the α chains. These observations are similar to those obtained by BORNSTEIN *et al.*[17]. Of importance was the fact that no major bands were seen between the α band and the buffer front, indicating that further cleavage of the collagen molecule at 25° did not occur.

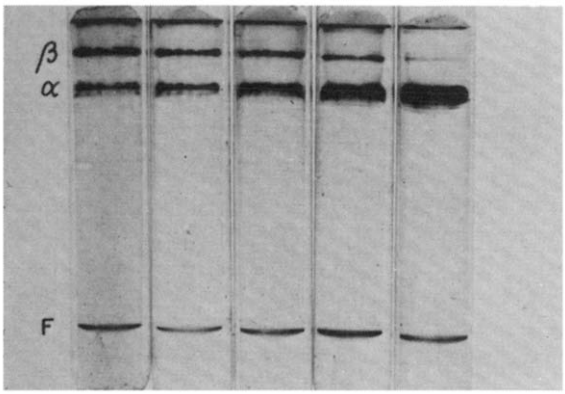


Fig. 6. Acrylamide gel electrophoresis patterns of chymotrypsin collagen reaction mixtures at 25°. From left to right: zero time reaction mixture and after 10, 20, 40 and 55% reduction in the initial specific viscosity. F refers to the buffer front.

Trypsin, at concentrations either equivalent to those present in a crude hepatopancreas extract, or equal to the amount of total protein in an aliquot of crude extract, had practically no effect on the viscosity of collagen in solution at 20–25°. In addition, even at a temperature of 25° trypsin does not alter the normal collagen pattern on disc electrophoresis.

Incubation of trypsin and chymotrypsin with ^{14}C-labeled collagen fibrils produces virtually no solubilization of radioactivity (Fig. 1) whereas, as noted previously, hepatopancreas extract results in extensive collagen degradation under these conditions.

Effects of inhibitors

The ability of various classes of inhibitors to block the action of hepatopancreas collagenase on native collagen fibrils was examined. EDTA has no effect on hepatopancreas collagenase, in contrast to the ability of this metal chelator to effectively inhibit bacterial[23], as well as other animal and human collagenases[6–10]. Cysteine, which blocks the action of bacterial, tadpole[6], and human-skin enzyme[9], has no effect on hepatopancreas collagenase. Diisopropylfluorophosphate and phenyl methyl sulfonyl fluoride, however, effectively inhibit hepatopancreas collagenase, but have no effect on these other collagenases.

DISCUSSION

The collagenolytic enzyme from the hepatopancreas of *Uca pugilator* fits into the broad definition of a collagenase[6] in that it is capable of degrading the polypeptide backbone of the collagen molecule under conditions that do not denature the protein. This is demonstrated by the ability of the enzyme to catalyze cleavages in the collagen helix resulting in a loss in viscosity of collagen solutions with no concomitant loss of optical rotation. In addition, essentially no dialyzable material derived from collagen is produced.

Since the hepatopancreas of the crab is a digestive organ, crude enzyme preparations, as might be expected, contain high levels of non-collagenase proteolytic activity. Approx. 1% of the protein in the crude extract is represented by an enzyme with chymotrypsin-like activity and one-third by a trypsin-like enzyme. The question of what role such levels of these enzymes might play in the degradation of collagen is of importance in distinguishing their action from that of the collagenase. Trypsin, at concentrations either equivalent to those present in crude hepatopancreas extract or equal in amount to the total protein content of an aliquot of crude extract, has practically no effect on the viscosity of collagen solutions at temperatures ranging from 20 to 25°. In addition, trypsin is ineffective in reducing the content of cross-linked components in native collagen[17,18,22]. Examination by disc electrophoresis of trypsin-treated collagen reveals no modified collagen fragments, demonstrating that the enzyme produces no significant cleavage of the collagen molecule. These data indicate that trypsin has little or no function in the primary degradation of collagen at 25°.

Chymotrypsin has a considerable effect on the viscosity of collagen but, like trypsin, is unable to produce cleavages in the native helix of the protein. The major action of chymotrypsin on collagen is the cleavage of peptide bonds from a nonhelical region at the N-terminal end of the molecule at or near the site of the intramolecular cross-link[17]. Although this results in the conversion of β components to α components, chymotrypsin has no activity on the main collagen helix and thus, is not capable of degrading the α chains further at 25°. It has been suggested[17,22] that the reduction in viscosity without the appearance of significant concentrations of new components reflects the ability of chymotrypsin to alter high molecular weight aggregates of collagen, resulting in solutions which are largely monomeric. It seems possible then, that the chymotrypsin-like activity present in the hepatopancreas extract is responsible in part for the early reduction in collagen viscosity and the conversion of β to α components. The presence of new fragments beneath the original α component (as well as below the original β component), observed on disc electrophoresis, most likely represent the products resulting from the action of the collagenase in the hepatopancreas extract.

Further evidence that these changes in the collagen molecule are due to the action of a collagenase was obtained from electron microscopic examination of segment-long-spacing crystallites precipitated from reaction mixtures. The presence of at least three modified segment-long-spacing aggregates indicates that the hepatopancreas collagenolytic enzyme has made several cleavages in the native collagen backbone, producing shortened collagen molecules which retain their native helical structure even in the presence of high concentrations of other proteolytic enzymes. Whether the crustacean hepatopancreas collagenase molecule possesses chymotryptic activity as well as collagenase activity cannot be established until the enzyme has been purified.

In addition to the data obtained on collagen in solution, it is important to note that trypsin and chymotrypsin, even in high concentrations, are unable to solubilize native fibrillar collagen (Fig. 1). Crude hepatopancreas extracts, however, are capable of producing extensive degradation of collagen fibrils, both in the form of reconstituted gels or whole tail tendon, again indicating the presence of a true collagenase in the hepatopancreas.

The collagenolytic enzyme from crab hepatopancreas is capable of attacking

the collagen molecule at a point one quarter the molecular distance from the "B" end, producing TC^{A}_{75}. It then continues to digest the cut end of TC^{A}_{75}, resulting in pieces approx. 70% (TC^{A}_{70}) and 67% (TC^{A}_{67}) the normal length of the molecule. It is not unlikely that these fragments represent the three major modified bands moving in front of the normal α component on disc electrophoresis. The conversion of β to α components apparently involves the removal of only a few amino acids, since "A" end modifications have not been observed in the electron microscope.

The possibility exists that the cleavage of the collagen molecule by hepatopancreas collagenase produces only two fragments, TC^{A}_{75} and TC^{B}_{25}, as is the case for most other animal collagenases[5,6,8-10] and that these fragments are then degraded by the action of other proteases in the extract. The fact that there is little or no loss in helical content following enzymatic attack by hepatopancreas collagenase and the finding of SAKAI AND GROSS[24] that tryptic action on fragments produced by tadpole collagenase results in a loss of optical rotation, makes this possibility seem less likely. Further, in view of the observation[24] that TC^{A}_{75} is not susceptible to tryptic attack at 10°, our finding that identical products are formed at 10 and 25° indicates that hepatopancreas collagenase itself catalyzes several cleavages in the collagen molecule.

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