Action of Proteolytic Enzymes on Collagen. (17698)

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In studies on comparative properties of collagens from various sources it was observed that samples prepared by mild processes were generally resistant to the action of common proteolytic enzymes with the exception of pepsin. Collagens prepared by more drastic means and commercial hide powder were highly susceptible to attack by the proteolytic enzymes. It was apparent that the use of gelatin, hide powder, or any modified collagen as a substrate for the detection of so-called "collagenase" activity would not be valid. In our laboratory *Clostridium histolyticum* filtrates and pepsin did actively solubilize collagen. *Clostridium perfringens* (*BP6K*) filtrates reputed by Maschmann(1), Macfarlane *et al.*(2), and Oakley *et al.*(3) to contain a "collagenase" were 10–20-fold less effective on collagens than equal quantities of *Cl. histolyticum* filtrates although hide powder and gelatin were attacked at a rapid rate. Jennison(4) reported that *Cl. perfringens* did not attack collagen from cattle tendon and cattle hide although his data are of a qualitative nature. It has been reported(5,6) that trypsin pretreatment results in greatly increased solubilization of collagens at the shrinkage temperatures (50-70°C). In the present work collagens from several sources revealed this effect after pretreatment with trypsin and papain. However, data are presented indicating that residual traces of enzyme are responsible for the increased solubilization. Heating native collagen in aqueous solution at 60-70°C renders it susceptible to tryptic action. In the present study it is shown that heat or urea denaturation of cattle Achilles tendon collagen results in increased digestion by trypsin, papain, and *Cl. perfringens* enzyme preparations. The increase in amount digested is approximately the same for all 3 enzymes.

Experimental procedure. Collagen† was prepared from Achilles and tail tendons of various animals by simple mechanical separation and shredding in the cold in a Waring Blendor with subsequent washing and extraction with cold acetone, alcohol, and ether. More highly purified material was attacked by proteases to the same extent. *Cl. histolyticum* and *Cl. perfringens* enzymes were produced in culture filtrates on a medium previously described(8). Incubation of collagen

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Kangaroo tail tendon by Johnson and Johnson Co., Brunswick, N.J., and by Dr. T. Salo, M. I. T. These were air-dried sinews.

Steer hide collagen by Dr. R. M. Lollar and Dr. Peter R. Buechler, Tanners' Research Council Laboratory, University of Cincinnati. Two samples were obtained: one prepared according to a published procedure(7) by treatment with $Ca(OH)_2$ and trypsin, and the second by brief extraction at 0°C with 10% NaCl and then with acetone.

Sodium "Lorol" sulfate PT, a wetting agent, was supplied by E. I. duPont de Nemours and Company, Wilmington, Del.

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TABLE I.
Solubilization of Collagens by Enzymes.*

Incubation time, hr	Enzyme: Trypsin, %	Cryst. trypsin, %	Papain, %	Cryst. chymotrypsin, %	*Cl. perfringens* pptd. enzyme, %	*Cl. perfringens* filtrates, %	*Cl. histolyticum* filtrates, %	Pepsin, %	
1	59.4	60.3	53.8	57.7	20.6	22.5	50.4	7.7	Hide powder
24								88.5	
1	5.4	2.3	3.3	1.4	0.0	0.0	2.0	0.0	Cattle Achilles tendon
24	8.2	1.8	3.0	4.3	12.4	18.9	51.4	94.7	
72	4.9	6.8	6.6	5.6	22.1	47.2	94.7		
1	3.8					1.8			Pig Achilles tendon
24	5.7					21.4	97.4	95.7	
72	5.0					63.1			
1		2.4	1.2	0.0	2.9†	0.0	1.3		Kangaroo tail tendon
24	4.9	1.2	2.2	3.3	6.9†	15.2	23.8	80.8	
72	7.9	4.4	5.4	4.4	18.4†	29.5	49.8		
1						1.1			Rat tail tendon
24	4.9				36.9†	43.6	91.8		
72					68.3†	87.3			

* Enzymes used in the following quantities:

Trypsin (Merck 1:250)	1.0 mg	*Cl. perfringens* pptd. enzyme	2.0
Crystalline trypsin	0.1	*Cl. perfringens* filtrates	3.0 ml
Papain (KCN activated)	1.0	*Cl. histolyticum* filtrates	0.2
Crystalline chymotrypsin	0.2	Pepsin (U.S.P.)	1.0 mg

† 5.0 mg.

TABLE II.
Solubilization of Collagen After Enzyme Pretreatment.

Treatment of Collagen after incubation with trypsin or papain			Collagen residue dissolved by heating, %
Washing agent	Heated at °C	Heating medium	
	Trypsin.*		
Distilled water	66	Distilled water	94
M/10 Na_2HPO_4	,,	,, ,,	14
,, NaH_2PO_4	,,	,, ,,	16
,, NaOH	,,	,, ,,	8
2% Na "Lorol" sulfate	,,	,, ,,	5
Distilled water	,,	0.1% H_2O_2	94
,, ,,	70	Distilled water	94
,, ,,	75	,, ,,	62
,, ,,	80	,, ,,	28
,, ,,	85	,, ,,	12
,, ,,	100	,, ,,	12
	Papain.†		
,, ,,	66	,, ,,	50-90
M/10 Na_2HPO_4	,,	,, ,,	49
,, NaOH	,,	,, ,,	5
Distilled water	75	0.01% H_2O_2	5
,, ,,	70	Distilled water	81
,, ,,	75	,, ,,	94
,, ,,	80	,, ,,	94
,, ,,	85	,, ,,	94
,, ,,	95	,, ,,	94
,, ,,	100	,, ,,	27

* 1 mg per tube.
† (activated with KCN) at 2 mg enzyme per tube.

with enzymes other than pepsin was conducted at 37° C in tubes containing 100 mg of substrate and 5 ml of 0.05 M phosphate buffer at pH 7.2. Papain was activated with 0.01 M KCN. Pepsin digestion was conducted in 10 ml of HCl solution at pH 2. A reasonably accurate measure of digestive action of enzymes was obtained by weighing the washed and dried residue of the collagen. Enhanced ease of solubilization of collagen at 66-75°C after enzyme pretreatment was determined after washing the collagen residue several times with distilled water or other solution. The residues were resuspended in water or other solution and placed in a water bath at designated temperature 20 minutes. The degree of solubilization was measured as before. Heat denaturation of a percentage of the collagen was accomplished by heating 100 mg in 5.0 ml of water at 65°C for 20 minutes. Urea denaturation of a percentage of the collagen was accomplished by suspension of 100 mg in 5.0 ml of 4 M, 5 M, or 6 M urea at 37°C for 24 hours. After denaturation the supernatant was removed and residues washed. The residues were then incubated with the enzymes.

Results. It is obvious as shown in Table I that hide powder is highly susceptible to attack by all the proteases tested. Extensive and rapid digestion of all collagen samples was obtained only with *Cl. perfringens* and *Cl. histolyticum* filtrates, and pepsin. Collagen preparations from chicken tendon, sheep tendon, turtle subcutaneous membrane, cattle tail tendon, rat tail tendon, kangaroo tail tendon, cattle *chordae tendinae,* cattle hide, and cattle bone showed resistance to trypsin and susceptibility to *Cl. histolyticum* enzyme. Other experiments not noted in the table demonstrated that collagen prepared by relatively drastic procedures and thus possibly modified was much more susceptible to digestion by all the enzymes, especially during the initial stages of the digestion.

Pretreatment with trypsin, crystalline trypsin, and papain increased the ease of solubili-

TABLE III.
Digestion of Cattle Achilles Tendon Collagen After Heat Treatment and Incubation with Urea.

Enzyme	Pretreatment of Collagen	Solubilization, %
Trypsin*	0	2.5
	20 min. at 65°C	68.2
Papain†	0	0
	20 " " "	68.2
Cl. perfringens pptd. enzyme‡	0	6.2
	20 " " 76	71.8
Trypsin*	Distilled water 24 hr at 37°C	3.3
	4 M urea " " " "	0
	5 " " " " "	35.9
	6 " " " " "	59.0
Papain†	Distilled water " " " "	1.4
	4 M urea " " " "	6.6
	5 " " " " "	31.6
	6 " " " " "	58.8
Cl. perfringens pptd. enzyme‡	Distilled water " " " "	16.4
	4 M urea " " " "	10.5
	5 " " " " "	43.4
	6 " " " " "	70.0

* Trypsin at 1 mg per tube.
† Papain (KCN activated) at 2 mg per tube.
‡ *Cl. perfringens* pptd. enzyme at 2 mg per tube.
Incubation with enzyme 24 hr at 37° C ± 0.5°.

zation of collagen at 66 to 75°C after thorough water washing. Crystalline chymotrypsin and *Cl. perfringens* enzymes did not show appreciable effect. As shown in Table II washing with M/10 solutions of phosphate or sodium hydroxide practically eliminated the solubilization effect. Na "Lorol" sulfate which inhibits trypsin destroyed the solubilization effect. At the same time peroxide did inhibit the solubilization due to papain but not that due to trypsin. Applying a post-enzymic heating treatment of 66-100°C to the collagen residues demonstrated an inhibition with rise of temperature. Collagen from different sources displayed these characteristic reactions. It appears that the reported ease of solubilization of collagen after trypsin pretreatment is an effect of quantities of enzyme which are difficultly removed from collagen by washing in water at room temperature.

Table III shows that heating collagen in water at 65°C or treatment with urea increased susceptibility to 3 different proteases to the same extent. Collagen from chicken tendon, pig tendon, sheep tendon, turtle subcutaneous membrane, cattle tail tendon, cattle *Chordae tendinae,* cattle hide, and cattle bone after similar heat treatment became susceptible to tryptic attack.

Summary. 1) The use of hide powder as a substrate in the demonstration of collagenase activity is not valid.

2) Collagens from several sources, prepared by methods designed not to alter properties, are resistant to the action of trypsin, chymotrypsin, and papain. The collagens are readily attacked (solubilized) by the proteolytic enzyme(s) of *Cl. histolyticum* and by pepsin. The proteolytic enzyme(s) of *Cl. perfringens* filtrates are 10–20-fold weaker than those of *Cl. histolyticum* filtrates in the degradation of collagen.

3) The reported increased solubilization of collagens in water at the shrinkage temperature (68–70°C) after incubation with enzymes can be attributed to residual enzyme.

4) Denaturation of collagen by heat and urea produces a general susceptibility to common proteolytic enzymes.

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