

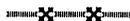
# ELASTASE ACTIVITY OF SOME ENZYMES AS RELATED TO THEIR DEPILATORY ACTION\*

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## ABSTRACT

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The elastase activity of some 15 enzyme preparations has been determined by three methods: formol titration, histological, and gravimetric. Results obtained with the three methods did not rate the enzyme preparations in the same order, nor is there any correlation of hair loosening with the elastase activity. A preparation of keratinase from *Streptomyces fradiae* exhibited the most potent elastase activity of any of the enzyme preparations tested.



## INTRODUCTION

This laboratory has been working for some time on an enzyme unhairing process that would be rapid and, in addition, would eliminate the problem of disposing of used lime-sulfide liquors, a problem which is becoming increasingly acute. Although considerable information has been obtained (1, 5, 8), the exact nature of the substance that binds the epidermis to the dermis is not known, and hence the identity of the enzyme or enzyme systems that cause the breakdown of this material remains obscure. Such information, if available, would be very useful in establishing an efficient enzyme unhairing system.

We have attempted to throw light on this reaction by assaying enzyme preparations for various activities and trying to correlate such activities with their hair-loosening ability. Earlier, it was shown that no correlation exists with starch-dextrinizing power and that only a casual relationship to casein digestion was demonstrable (1). Among the components of the system which have been suggested as anchoring material for the epidermis is the fibrous protein elastin (2, 3). If a strong correlation between elastase activity and hair-loosening ability could be shown, it would lend credence to this view.

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## EXPERIMENTAL

### *Materials*

*Hide* samples were all from green-salted hides obtained from a local slaughter house. On arrival at the laboratory they were fleshed and stored in a refrigerator in moisture-proof bags.

*Enzymes* were obtained from commercial sources and were used without further purification. They are listed and described in Table I.

TABLE I  
ENZYME PREPARATIONS USED IN ELASTASE ASSAYS

Enzyme and Supplier	Source
<i>Miles Chemical Company, Clifton, N. J.</i>	
HT Proteolytic (extra high potency)	Bacterial
HT Concentrate (market grade)	"
HT Proteolytic 200	"
Special Diastase 160	Plant and Animal Materials
Bromelin	Pineapple
<i>Rohm &amp; Haas, Philadelphia, Pa.</i>	
Protease 15 Concentrate	Bacterial
Rhozyme P11	Fungal
Lipase B	Microbial
<i>Wallerstein, Inc., New York, N. Y.</i>	
Protease 4511-3	Bacterial
Prolase 40	Fungal
<i>Nutritional Biochemicals Corp., Cleveland, Ohio</i>	
Trypsin Non-Crystalline 4 x USP	Pancreas
Papain	Plant
<i>Pabst Laboratories, Milwaukee, Wisconsin</i>	
Protease L-56-D	Bacterial
<i>Viobin Corporation, Monticello, Illinois</i>	
Viokase	Pancreas
<i>Merck Sharp and Dohme, Rahway, New Jersey</i>	
Keratinase	<i>Streptomyces fradiae</i>

*Elastin* for use in the formol titration procedure was that purified from *ligamentum nuchae* obtained from the Worthington Laboratories\*.

\*Mention of a company and/or product name by the United States Department of Agriculture does not imply approval or recommendation of the company or product to the exclusion of others not mentioned.

### Methods

*Formol titration method.*—This is the standard method for determining carboxyl groups resulting from hydrolysis of the peptide links, by enzymes in this case. Formaldehyde is used to bind the amino groups, and the carboxyl groups are titrated. Elastin was treated with three concentrations of the enzyme at different pH levels for 4 hours at 30°C. on a shaker. After the addition of formaldehyde the solution was titrated with standard alkali. The control was run in the same way except that the enzyme was added after the formaldehyde. Samples were titrated with 0.1*N* NaOH using phenolphthalein indicator.

*Histological and gravimetric methods.*—Two-inch-square pieces of hide were immersed in 2% enzyme solutions for 7 or 17 hours at 38°C. This concentration is about ten times that required for unhairing. It was selected to make certain that lack of enzyme would not be a limiting factor in periods of one working day, or overnight plus one working day. Control pieces from the same hide were immersed in 5% salt solution until the hair and epidermis could be removed, which required up to 2 weeks. Bacterial growth was prevented by adding 0.015% phenylmercuric acetate to the solutions. The hair and epidermis were removed from all samples, and they were thoroughly washed. Samples for histological assay were fixed in formalin and sectioned, stained, and evaluated as described by Everett *et al.* (4).

For the gravimetric assay the enzyme-treated hide pieces and the controls were autoclaved in 200 ml. of distilled water at 15 lb. pressure for 4 hours to dissolve the collagen. The solutions were decanted through tared, fritted-glass crucibles, and the residues were washed once with hot water. The residues were again autoclaved, transferred to glass crucibles, washed consecutively with ethyl alcohol and acetone to remove the fat, and dried. Results are reported as "percent less than control."

*Unhairing activity.*—The comparative hair-loosening activity of most of these enzymes has been reported previously (1), and the method of measurement has also been given (1, 5). Essentially it consisted of pulling a 1" blade repeatedly over the treated hide until no further hair was removed. The number of pulls and the estimated percent of hair removed were recorded. In this paper a numerical value for hair loosening has been obtained by dividing *R*, the estimated percent of hair removed, by the number of pulls *P*, multiplied by the enzyme concentration in solution as percent.

### RESULTS

Data obtained with several enzymes, at two pH levels in most cases, using the formol titration procedure, are plotted in Fig. 1. The highest values, i.e., the most acid produced, were obtained with a bacterial protease

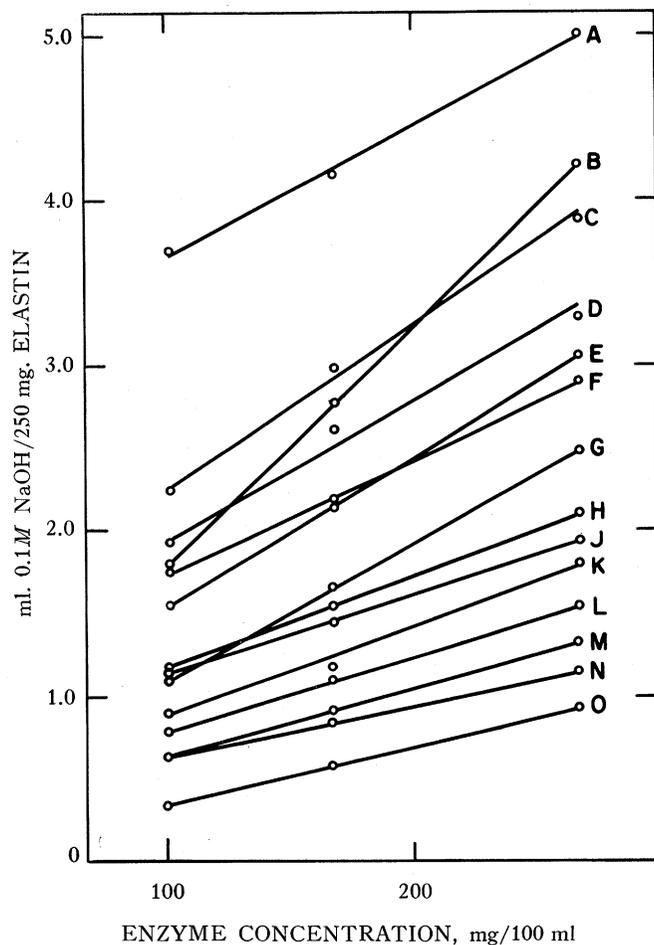


FIGURE 1.—Relationship between enzyme concentration and titratable acid produced from prepared elastin in 4 hours at 30°C.

A.	HT Proteolytic P	pH 6.5
B.	HT Proteolytic P	pH 8.8
C.	HT Concentrate 4903	pH 6.5
D.	Protease 15 Concentrate	pH 6.5
E.	HT Concentrate 4903	pH 8.8
F.	Protease L56-D	pH 6.5
G.	Viokase	pH 8.8
H.	Protease 4511-3	pH 6.5
J.	HT Proteolytic 200	pH 7.0
K.	Trypsin Non-Crystalline	pH 8.8
L.	HT Proteolytic 200	pH 8.8
M.	Viokase	pH 7.0
N.	Viokase	pH 6.5
O.	Rhozyme P11	pH 6.5

at pH 6.5. At pH 8.8 the activity with this enzyme was considerably less, although the slope of the curve was steeper, indicating a decreased pH effect at the higher concentrations of enzyme. All the bacterial proteases were more active at neutrality or slightly below than at pH 8.0 or above. The pancreatic enzymes were more active on the alkaline side, as expected. The two fungal enzymes showed only very weak activity.

The histological procedure permits an evaluation of the general attack on the hide constituents by the enzymes. The effect of pH on general proteolysis, as well as elastolysis, is shown in Table II for the enzyme preparation HT Proteolytic. Controls without enzyme were run concurrently, but only

TABLE II  
EFFECT OF pH ON ELASTOLYSIS AND GENERAL PROTEOLYSIS  
OF HIDE BY HT CONCENTRATE 4903  
(2% Solution, 7 hr., 38°C.)

pH	Elastolysis		General Proteolysis*	Condition of Epidermis
	Elastin Degraded %	Completely Cleared %		
4	0-10	0	weak	separated
5	10-25	0	weak	separated
6	20-40	0	moderate	mostly gone
7	50-75	10-20	strong	gone
8	40-60	0-10	strong to severe	gone
9	40-60	10-20	strong	gone
10	30-50	0	strong to severe	gone

\*Attack on cells, glands, muscle fibers, etc.

very mild action at the pH extremes was observed. With HT Proteolytic, the strongest attack on elastin was at pH 7.0, but strong action continued even at 10.0. It is interesting that complete elimination of elastin occurred to a depth of only 20% of the papillary layer and then only at the pH of maximum activity. General proteolysis, observed as attack on glands, muscles, capillaries, etc., was strongest at pH 8.0 and above. The epidermis was separated from the dermis even at pH 4.0. The epidermis was almost completely disintegrated at pH 6.0 and disappeared completely at pH values above this point.

Results obtained with 14 enzyme preparations using the three different methods of assay are compared in Table III. Also included are results of the unhairing activity of these enzyme preparations. According to the formal titration procedure, the top-ranking preparations are of bacterial origin. Bromelin also ranks high by this method. In the histological method, Vio-

TABLE III  
RELATION OF THE ELASTASE ACTIVITY TO HAIR-LOOSENING ACTION OF 14 ENZYME PREPARATIONS

Enzyme	Elastase Assay Method													Hair Loosening*				
	Formol Titration				Histological				Gravimetric					Enzyme Concentration %	P**	R††	(R/P) x Conc.	Rank
	pH	Meq./g.†	Rank	Incubation Time	Elastin Degraded %	Rank	pH‡	Percent Less Than Control	Rank	Enzyme Concentration %	Rank	Rank						
HT Proteolytic P Concentrate	6.5	12.5	1	7	75-100	2	6.7	79.0	3	0.06	2	99	825	1				
HT Concentrate	6.5	9.8	2	7	50-75	4	6.6	70.5	5	0.10	3	99	330	2				
Protease 15	6.5	8.3	3	17	30-50	13	6.3	70.0	6	0.12	6	90	125	5				
Protease L-56-D	6.5	7.3	4	17	30-50	13	6.1	49.0	12	0.15	3	99	220	3				
Bromelin	5.2	7.0††	5	7	60-80	3	5.1††	53.5	10	0.41	3	98	79	8				
	8.8	none																
Viokase	5.5	none		7	50-70	5	7.4	70.0	6									
	8.8	6.3	6	7	80-100	1	8.6	74.0	4	0.38	10	95	22	11				
	6.5	2.9		7	60-80	3	7.3	83.0	2									
	5.5	none		7	30-50	8	5.7	55.5	9									
HT Proteolytic 200	7.0	5.0	7	7	10-30	11	6.7			0.21	3	95	151	4				
Protease 4511-3	6.5	5.0	7	17	70-90	12	6.4	51.5	11	0.37	3	98	88	7				
Trypsin (4 x USP Pancreatin)	8.8	4.5	8	7	50-70	5	8.7	88.0	1	0.26	3	95	122	6				
				7	30-50	8	7.4	67.5	7									
Papain	8.8	3.5***	9	7	20-40	10	5.8	38.0	15									
	5.5	none		7	30-40	9	7.3	6.5	19	0.73	6	97	22	11				
				7	40-70	6	5.5	33.0	16									
Rhozyme P-11	6.5	2.3	10	7	50-60	7	5.2††	57.0	8									
Prolase 40	5.5	1.0	11	17	20-30	14	6.4	39.0	14	0.78	3	98	42	9				
Special Diastase 160	6.5	0.0		17	0-5	16	5.9	44.0	13	0.83	4	98	30	10				
Lipase B	6.5	0.0		17	5-20	15	6.1	9.5	18	2.59	2	99	15	12				
				17	0		6.3	28.5	17	10.0	10	0						

\*From Cordon *et al.* (1,5).  
 \*\*Number of pulls with scraper.  
 \*\*\*With BAL as activator.  
 †Enzyme concentration, 0.27%.  
 ‡Estimation of percent hair removed.  
 ††Applies to histological and gravimetric determinations.  
 †††With 1% sodium metabisulfate.

kase at pH 8.6 was most active; a bacterial protease, HT Proteolytic P, was next, and bromelin and Viokase (pH 7.3) were tied for the third position in activity. According to the gravimetric assay, trypsin at pH 8.7 ranked first; Viokase at pH 7.3, second; and HT Proteolytic P, third.

Bromelin showed no activity by the formol titration procedure unless an activator such as sodium metabisulfite was present. This reducing agent also increased the activity of bromelin in the histological assay.

Papain was not active according to the formol titration assay even in the presence of sodium metabisulfite, but 2,3-dimercaptopropanol (BAL) produced considerable activation.

Rhozyme P11 and Prolase 40, two fungal enzymes, and special Diastase 160, which is a mixture of plant and animal enzymes, were among the weakest in all the materials assayed. Lipase B showed activity only in the gravimetric assay, and this was very weak.

Since the completion of this work with these enzymes another enzyme preparation possessing strong depilatory activity has become available. It is produced by *Streptomyces fradiae* and was discovered, and named keratinase, by Nickerson and Noval (6, 7). It is now under commercial development. Since the work with this enzyme was, of necessity, carried out on a different hide, the results are reported separately.

As with the other enzymes, a high concentration, roughly ten times that needed for unhairing, was used to insure that the amount of enzyme would not be a limiting factor and that activity would be evident in a short incubation period. The strength of this enzyme has been determined by an assay procedure in which wool is used as the substrate, and absorption in the supernatant at 280 m $\mu$  is measured (7, 8). The same assay procedure has been used in this work. About 100 to 150 keratinase units per ml. of solution are

TABLE IV

ELASTASE ACTIVITY OF KERATINASE FROM *STREPTOMYCES FRADIAE*

Concentration Ku/ml	Time hr.	pH	Additive %	Histological		Gravimetric
				Depth Degraded %	Depth Cleared %	Percent Less Than Control
1500	7	6.8	none	75	45	56
1500	7	8.8	Borax, 0.5	100	100	84
1500	7	8.7	Borax, 0.5			
			NaCl, 5.0	60	35	85
136	22	8.7	Borax, 0.5	100	100*	
136	22	8.5	Borax, 0.5	100	100**	
			NaCl, 5.0			

\*No elastin in entire thickness of hide  
\*\*Many fragments in center corium

required for good depilatory action. Accordingly, 1500 units per ml. were used in this study. Table IV gives the results obtained with the three assay methods when the pH was left unadjusted (pH 6.8), with borax added to raise the pH to 8.8 (approximately the optimum for unhairing action), and with 5% sodium chloride present in addition to the borax. Strong elastase activity was evident even at the low pH of 6.8 and was very strong at 8.8. The effect of sodium chloride in the enzyme solution was tested because it appears to have a beneficial effect on the physical characteristics of the hide and possibly of leather made from it. As shown, it decreased the elastase activity, as measured histologically, but the elastin was affected in some manner because it was hydrolyzed by the hot water in the gravimetric determination. Elastase activity was determined histologically on some bends which had been treated with keratinase at 136 K units/ml. in a rocker vat for 22 hours at 40°C. Without salt in the enzyme solution there was no trace of elastin remaining throughout the entire cross section of the hide. This is the only time we have observed such complete removal of elastin. With sodium chloride present, many fragments of elastin still remained in the center of the corium, but the grain and flesh areas were completely cleaned out.

TABLE V  
ELASTASE ACTIVITY OF HT PROTEOLYTIC E 210 IN THE  
PRESENCE AND ABSENCE OF SODIUM CHLORIDE

Concentration %	Time hr.	pH	Additive %	Histological		Gravimetric
				Depth Degraded %	Depth Cleared %	Percent Less Than Control
2.0	7	6.6	none	40	13	53
2.0	7	8.7	Borax, 0.5	55	15	49
2.0	7	8.8	Borax, 0.5			
			NaCl, 5.0	18	0	61

Sodium chloride was shown to exert a similar effect on the elastase activity of the bacterial protease HT Proteolytic, Table V. From Table V it appears that the elastase activity of this enzyme was inhibited by salt when measured histologically but was unchanged when measured gravimetrically.

From the data for hair loosening, it is seen that of the seven most active preparations, six are of bacterial origin. (Although not included in this comparison for reasons stated above, keratinase is also a very potent hair-loosening agent). The elastase assay most nearly agreeing with this ranking is the formol titration, but bromelin and Viokase are included in place of two of the bacterial preparations, and only the first two agree as to order. Certainly no good correlation exists between hair loosening and any of the assays. Furthermore, the assays do not agree with each other.

## DISCUSSION

As far as is known, the use of the formol titration procedure for determining elastase activity has not been successfully employed before. Banga, in an attempt to measure pancreatic elastase activity, is reported by Thomas and Partridge (9) to have been unable to detect the release of amino groups by this method. This method has the advantage over several others in that it is direct and measures actual breakdown of tissue, i.e., rupture of peptide bonds. The splitting of dye molecules from stained elastin as in the method of Sachar *et al.* (10) might not be indicative of elastin breakdown unless supported by other data. Any method utilizing isolated elastin suffers from the fact that the elastin is undoubtedly changed by the isolation procedure. In this respect the gravimetric method used, or a very similar one employed by Ornes and Roddy (11), is superior. However, the difficulty of obtaining suitable controls with the notoriously nonuniform hide is objectionable. Furthermore, any method which measures the unidentified residue of a reaction is open to the criticism that the residue may not consist wholly of the constituent in question. In this case keratin, fat, etc. may not be completely removed.

Histological studies are certainly of great value, but interpretation of results must be made with caution. It was found that the elastin in lime-sulfide unhaired hide was stained in almost characteristic manner with elastin stains. However, the elastin had been attacked, as shown by the fact that when this limed hide was autoclaved, about half of the elastin was dissolved. Banga *et al.* (12) have reported an analogous situation. The transparency of elastin particles increased through enzyme action, although dissolution of elastin could not be demonstrated until later.

The use of intact tissue for elastase assay has the advantage that the elastin is not altered by preparatory procedures. It appears, however, that other components of the hide influence the enzyme action. Neither bromelin nor papain showed any activity on isolated elastin without an activator, whereas on intact hide there was considerable action without addition of activators.

Although some of the best depilatory enzymes were also high in elastase, no high degree of correlation between these two actions could be demonstrated. The results indicate that elastin does not contribute to the anchoring of the epidermis. Therefore, measurement of elastase activity would not be of any assistance in determining the unhairing action of enzymes.

## SUMMARY

Three methods of determining elastase activity have been described. Elastin activity on isolated ligament was measured by a formol titration procedure. Gravimetric and histological methods were used on intact hide. Agreement among the three methods was not good. Elastase activity of the

enzymes did not correlate well with their depilatory action. Keratinase from *Streptomyces fradiae* shows the strongest elastase activity of any of the enzymes tested.

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#### DISCUSSION

DR. JAMES CONSTANTIN (Merck and Company): We are again indebted to Dr. Cordon and his associates at the Eastern Regional Research Laboratory for another fine paper on the methods and measurements of enzymatic unhairing.

In my opinion Dr. Merrill, I think in "The Chemistry and Technology of Leather", states the problem of enzymatic unhairing fairly when he says that we must know how enzyme X acts on substrate Y, forming product Z.

As to the question of enzyme X we can identify it by a number of assay methods. Over the last few years, Dr. Cordon's work on the effect of various enzymes on substrate Y has been most interesting.

Dr. Cordon, you have mentioned in the course of your discussion starch dextrinization, gelatin viscosity, and casein hydrolysis as measures of protease activity. Can you elaborate on other methods that you have used in your search for a method of enzyme unhairing?

DR. CORDON: We have investigated their action on gelatin. We find hair-loosening activity associated with strong proteolytic enzyme. But we do not find a high correlation between depilatory action and gelatinase activity by any of the three procedures we have used.

DR. CONSTANTIN: In a meeting of the Society of American Bacteriologists in May, Dr. Cordon, you also mentioned the keratinase assay of Noval and Nickerson, and if it were reported to me correctly, you found a fairly good correlation between enzyme activity and this keratinase assay. But once again it was the same type of correlation, wasn't it?

DR. CORDON: Yes. That is true. We have not compared the depilatory action with the keratinase activity. But I understand you have, and I know Dr. Nickerson claims that he has followed it. I am sure this assay would not be applicable to other enzymes. We have tested bacterial enzymes by the keratinase assay, and although they are strong depilatories, they have very little action on the wool, according to this procedure.

DR. CONSTANTIN: From reading some of your other papers, and from a quick comparison of the 2% level of enzyme that you mentioned, and also considering the somewhat higher temperatures used (40°), we know as a rule of thumb that enzyme activity will roughly double for every 10° C. rise in temperature. Actually then, doesn't this mean that the level of enzyme that you used in these elastinase experiments is something like twenty times the level that you need for effective unhairing?

DR. CORDON: Yes, I think that is correct. As I mentioned in the paper, we were not interested in unhairing in this case. We wanted to measure the effect these enzymes had on the elastin, and we wanted to produce the effect in one day without having to come back to the laboratory in the middle of the night. We put enough enzyme in so that the reaction would be fairly advanced in seven hours or, if not, so that observations could be made in 17 hours.

DR. TURLEY: I am not surprised that you find no correlation between the elastase activity and depilation. I wonder where the idea came from that the elastin fibers might be penetrating into the epidermis. In all the classic pictures of skin on the bodies of animals, it always seems that the elastin is anchored within the corium itself. I did not see it in your pictures, but if you go to the edge of the epidermis and the grain membranes, you will find elastin fibers growing smaller and smaller, but never do they seem to penetrate into the epidermis. So I am not surprised to find no correlation between elastase activity and depilation.

DR. CORDON: I found two references in medical literature in which it was claimed that elastin does play a role in anchoring the epidermis to the dermis, but our results do not point to that conclusion.

DR. BUECHLER: In your histological studies, Dr. Cordon, you measured the depth of penetration of the enzymes from the grain surface. Did you also find any evidence of penetration through the sides of the hair follicles?

DR. CORDON: That would be very difficult to determine. As I mentioned, fragmentation of the elastin fibers occurs first. Complete clearing out as the enzyme penetration progresses does not take place. First, voids appear in the fibers, and then just fragments are left. I believe, however, that the enzyme does penetrate through the hair follicles.

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