BBA 12252

KERATINASE

II. PROPERTIES OF THE CRYSTALLINE ENZYME

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(Received December 5th, 1962)

SUMMARY

Keratinase conjugate elaborated by *Streptomyces fradiae* has been resolved into a basic protein and a novel acidic polymer. The basic protein, obtained in crystalline form, exhibits maximum proteolytic activity against wool at its isoelectric point (approx. pH 9.0). Crystalline keratinase was shown to be homogeneous by electrophoretic and ultracentrifugal analysis. No oligopeptide, other than a hydrolytic product of poly-L-lysine, has yet been found to serve as substrate for keratinase. Properties of keratinase are compared with those of certain other microbial proteases. The nature of the linkage between protein and the acidic component of keratinase conjugate is considered.

INTRODUCTION

The keratin-digesting enzyme elaborated by *Streptomyces fradiae* occurs in culture filtrates as an enzyme conjugate consisting of a highly acidic, non-proteinaceous component coupled to a basic protein. The enzyme conjugate is stable under a wide range of conditions, and has been obtained in highly purified form by concentration, repeated ammonium sulfate precipitation, dialysis, and lyophilization. Keratinase conjugate was observed[1] to have a sharp optimum (for activity) at pH 9.0, and to exhibit minimum solubility at pH 3.6.

In the course of efforts to purify this enzyme, use was made of cellulose ion-exchange columns. On DEAE-cellulose, the conjugate was split into two components. Acidic material was retained on the column, and the protein component was obtained in the eluate. Wool-digesting activity was associated with the protein, and not with the acidic component. After concentration and dialysis of eluates from cellulose columns, a crystalline protein was obtained that exhibited marked wool-digesting activity. This crystalline enzyme has been termed keratinase in view of the fact that approximately one-third of the weight of essentially undenatured wool can be solubilized on incubation with the enzyme at 37° in pH 9.0 buffer for 24 h. Crystalline keratinase appears homogeneous on electrophoretic and ultracentrifugal analysis. Whereas the pH for optimal activity of the crystalline enzyme remains sharp at 9.0, its isoelectric point (minimal solubility and electrophoretic mobility) is shifted markedly, from that of the enzyme conjugate, to pH 8.95.

Abbreviation: AM, purified acidic material.

METHODS

Concentrated fermentation broths resulting from the growth of *Streptomyces fradiae* in nutrient media containing keratinaceous material were prepared as previously described[1]. Purified preparations of keratinase conjugate were subjected to further fractionation on ion-exchange cellulose (DEAE-cellulose, 0.96 mequiv/g), obtained from the Brown Co., Berlin, N. H. (U.S.A.).

The protein content of eluates obtained by column fractionation was determined by the method of LOWRY *et al.*[2] employing standard solutions of bovine serum albumin (Fraction V, Armour and Co.). Proteolytic activity of such eluates was determined by the procedure of ANSON[3] with denatured hemoglobin as substrate (Hemoglobin substrate powder, Worthington Biochemical Corp., Freehold, N.J.), and by the wool assay previously described[1]. Enzymatic hydrolysis of poly-L-lysine·HBr (Schwarz BioResearch, Inc.) was examined by the procedure of WALEY AND WATSON[4]; the solvent system they recommended (*n*-butanol–acetic acid–water–pyridine, 30:6:24:20, v/v) for separation of oligopeptides of lysine was employed. Trypsin (2-times crystallized, salt free) and soy-bean trypsin inhibitor (5-times crystallized) were obtained from Worthington Biochemical Corp. Tris, purified titrimetric standard (Fisher Scientific Co.) was employed as buffer in all experiments, unless otherwise mentioned. For determination of the pH optimum of crystalline keratinase, a buffer covering the range 7.5 to 10.0 was prepared with lysine·HCl (Schwarz BioResearch, Inc.) and NaOH.

RESULTS

Resolution of keratinase conjugate

DEAE-cellulose (32 g) was slurried with 500 ml of 0.01 M Tris buffer (pH 7), and 20 ml of concentrated fermentation broth was added. The mixture was agitated for a few minutes, then let stand for 1 h at 20°. The dark brown mass was filtered with suction through Whatman No. 2 paper to yield an amber-colored filtrate. The dark brown cellulose residue was washed with 450 ml of 0.01 M Tris buffer (pH 7), yielding a light yellow filtrate; a second wash with 300 ml of Tris buffer gave a pale yellow filtrate. The three filtrates were combined, and 15 g of DEAE-cellulose were added. This slurry was stirred for a few minutes, let stand for 10 min at 20°, then filtered with suction through Whatman No. 2 paper. The filtrate, deep lemon-yellow in color, showed a blue fluorescence when illuminated with light of 3650 Å. The filtrate was concentrated *in vacuo* in a rotating-film evaporator to 190 ml.

A column 2.5 × 91 cm was packed with a slurry of DEAE-cellulose (42 g) in 1100 ml of 0.01 M Tris buffer (pH 7), and washed with 500 ml of the same buffer. The concentrated filtrate (185 ml) described above was placed on the column and eluted with Tris buffer (pH 7). Fractions of 10 ml were collected at a flow rate of 1.5 ml/min. Through tube No. 28 the eluate was colorless, a yellow-orange fraction then commenced and continued through tube No. 59; after this point the color changed to a lemon-yellow color that showed a blue fluorescence when illuminated at 3650 Å. The latter color continued until tube No. 97. Elution with Tris buffer (pH 8.0) was begun at tube No. 98 and continued until tube No. 150; after tube No. 170 the Tris buffer was made 0.05 M in NaCl. These fractions, and others obtained with subsequent treatments, were assayed for protein content and for pro-

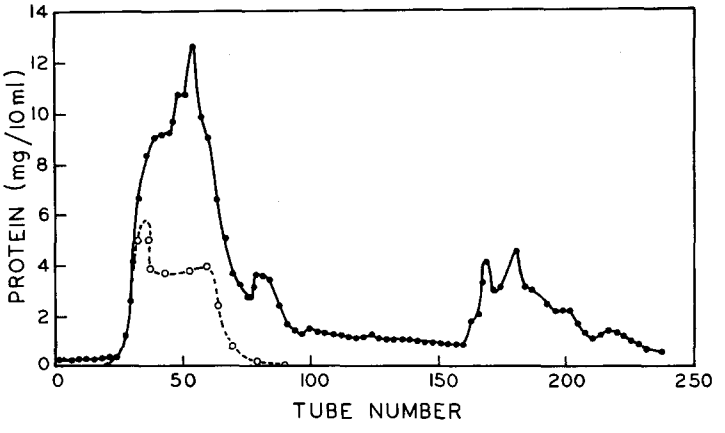

Fig. 1. Elution of protein (solid line) and proteolytic activity (dotted line) from DEAE-cellulose column. Details given in text; proteolytic activity expressed as mg protein/10 ml with reference to protein content of a standard preparation (K4A).

teolytic activity (Fig. 1). Proteolytic activity was associated with protein that was rapidly eluted from the column with Tris buffer (pH 7). Of the total proteolytic activity applied to the column, 56% was recovered in tubes Nos. 31–58. Proteolytic activity was not found in any fractions other than those of the initial protein peak. An example of the activity of one fraction against wool is shown in Table I.

TABLE I

ACTIVITY OF FRACTION* FROM DEAE-CELLULOSE COLUMN AGAINST WOOL**

Incubation time (h)	*ΔA at 280 mμ*	*Protein solubilized*		
		280/260 mμ		*Ninhydrin***
		mg	*Per cent of total*	*(mg)*
16	1.03	24.4	14.4	7.01
20	1.22	28.5	16.8	8.37
37.5	1.50	35.6	20.9	10.23
56.5	1.64	37.6	22.1	10.82
126.5	1.74	40.1	23.6	

* 1 ml of tube No. 36, containing 0.84 mg protein/ml.
** All values given on a cumulative basis.
*** Values expressed with reference to leucine standard.

Crystallization of keratinase

Advantage was taken of the fact that proteolytic activity was associated with fractions rapidly eluted from DEAE-cellulose by Tris buffer (pH 7). To obtain the enzyme in crystalline form, relatively large amounts of keratinase conjugate were contacted with the cellulose in bulk. DEAE-cellulose (80 g) was washed with 1500 ml of 0.05 M Tris buffer (pH 7), let stand for 30 min and filtered through Whatman No. 4 paper. The packed cellulose cake was slurried in 1 l of the Tris buffer and 5 g of purified keratinase conjugate (lot K5A), dissolved in 250 ml of Tris buffer, was added

to the slurry; an additional 250 ml of Tris buffer was added and the mixture was well stirred. The slurry was filtered through Whatman No. 4 paper; the cellulose cake was resuspended in 1 l of Tris buffer and again filtered; the cake was washed on the filter with 500 ml of buffer, and the two filtrates were combined. DEAE-cellulose (20 g) was added to the combined filtrates, slurried, and filtered as before to give 3 l of filtrate. The filtrate was concentrated *in vacuo* in a rotary evaporator at 37–40° to small volume. The concentrate (70 ml) was dialyzed against running distilled water for 12 h, then dialyzed at 4° against 1 l of distilled water for 48 h with toluene added to the dialysis sac as preservative. At the end of this time, three layers of material had formed at the bottom of the dialysis sac—a fluffy top layer, a coarse brown middle layer, and a white crystalline bottom layer. The supernatant liquid was carefully removed and the three layers were separated by means of pipette and syringe. After standing at room temperature for about 30 min, the supernatant fluid deposited a heavy white precipitate that was separated at 5000 rev./min in a refrigerated centrifuge.

The various fractions were examined under a polarizing microscope and had the following appearance: Q-F (fluffy), largely amorphous, a few brushes (polarizing); Q-B (brown), largely brushes (polarizing) and some amorphous material; Q-W (white), chiefly brushes (polarizing) and a few plates (polarizing); Q-S(precipitate from supernatant), chiefly radial clusters of rods (polarizing). Photographs of the several crystalline forms are shown in Fig. 2. The above four fractions were packed by centrifugation at 7000 rev./min, washed twice with 0.5-ml portions of distilled water by resuspending and centrifugation, then stored at −20° as pastes.

Proteolytic activity of the several fractions was assayed against hemoglobin substrate (Table II). Approximately one-half of the proteolytic activity present in the concentrated eluate from DEAE-cellulose was recovered in solid form. Crystalline

TABLE II

PROTEOLYTIC ACTIVITY OF FRACTIONS OBTAINED DURING CRYSTALLIZATION OF KERATINASE

Fraction	*Proteolytic activity* (K-units × 10^5)*	*Per cent of original activity*
Q-series		
Concentrated eluate (70 ml)	15.53	100
Amorphous (Q-F)	1.54	10.0
Brown crystalline (Q-B)	2.02	13.0
White crystalline (Q-W)	1.90	12.2
Crystalline (Q-S)	1.97	12.7
Residual liquor	3.42	22.0
Activity in solid form	7.43	47.9
T-series		
Concentrated eluate (70 ml)	2.14	100
Crystals Ta	1.00	46.9
Crystals Tb	0.65	30.5
Residual liquor	0.10	4.8
Activity in solid form	1.65	77.4

* Assayed against hemoglobin by method of ANSON[3].

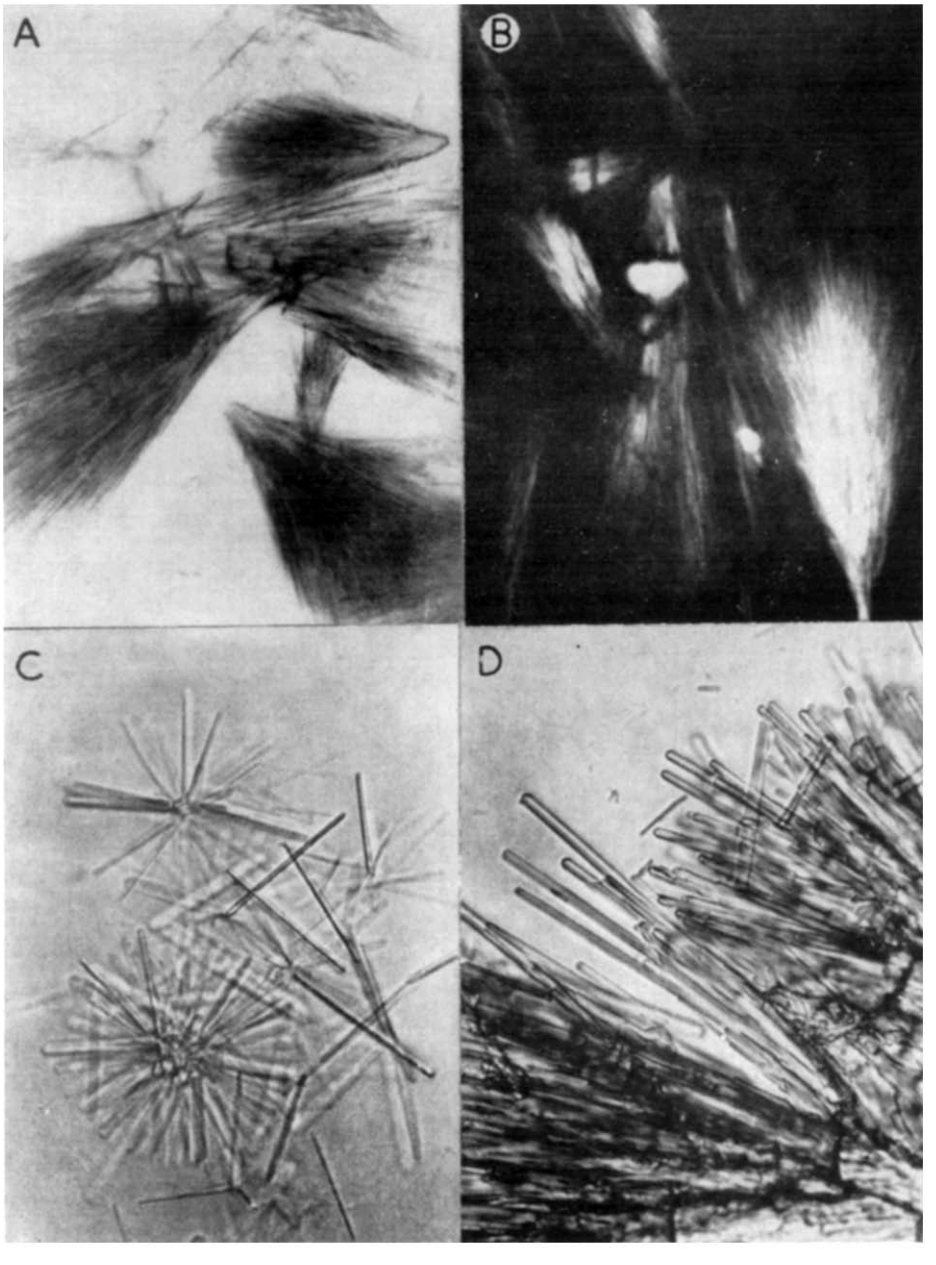

Fig. 2. Photomicrographs of crystals of keratinase. A, appearance of slowly formed crystals (brushes, 300 ×); B, same under crossed polarizers (600 ×); C, radial clusters of more rapidly formed crystals (300 ×); D, same 645 ×.

preparation Q-S had a specific activity of 4380 K-units/mg. This may be compared with the activity (475 K-units/mg) of a purified preparation of keratinase conjugate (K4A).

The crystalline form of the enzyme: "brushes" or radial clusters, appeared to depend upon the rapidity of crystallization. Rapidly crystallized enzyme always took the form of rods, generally arranged in radial clusters.

With one modification (noted below) the procedure for preparation of the crystalline Q samples was followed for all subsequent large-scale crystallization of enzyme. After DEAE-cellulose treatment, concentration, and dialysis against running distilled water for 12 h, the sample was dialyzed against 1 l of distilled water at 4° for only 24 h. At the end of this time a small amount of material, mostly amorphous, had collected in the bottom of the dialysis sac. This material was centrifuged off and the clear supernatant fluid was then dialyzed for 1 h against 1 l of 0.05 M Tris buffer (pH 9.0), approximating the isoelectric point of the crystalline enzyme (see below). Within 5 min a heavy white deposit had formed, which, on microscopic examination, was found to be crystalline and identical in appearance with crystalline keratinase sample Q-S. Crystalline samples (T-series, Table II) obtained by this procedure showed high specific activity when assayed against hemoglobin or wool substrates, and good recovery of the activity originally present was obtained.

Properties of crystalline keratinase

As shown in Fig. 3, the pH optimum of keratinase lies at 9.0, and the pH–activity curve resembles that previously obtained with the enzyme conjugate. To determine the effect of pH on the solubility of keratinase, a series of buffers (0.10 M) covering the pH range from 1.15 to 10.59 were prepared; KCl–HCl buffer from pH 1.15 to 2.13, glycine–HCl from pH 2.52 to 3.33, acetate buffer for pH 4.05 to 5.35,

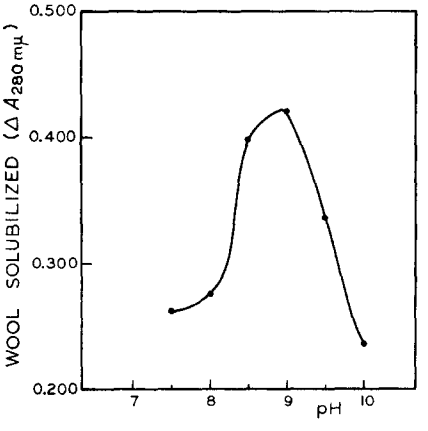

Fig. 3. Effect of pH on activity of crystalline keratinase against wool. Preparation Tb (20 μg/ml) employed in standard assay procedure; lysine·HCl–NaOH buffer (0.05 M).

phosphate buffer for pH 5.99 to 7.95, and glycine buffer for pH 8.53 to 10.59. To 1 ml of buffer was added 0.05 ml of a homogeneous suspension of enzyme (preparation Q-S) in water. The tubes were held at 4° for 10 min. Contents of tubes in the range pH 1.15 to 6.52 were clear, tubes from pH 6.95 through pH 9.48 were turbid, and those at pH 10.00 and 10.59 were clear. The contents of the tubes were diluted

to 3.0 ml with water and turbidities estimated spectrophotometrically at 400 mμ. Minimum solubility was evident at pH 8.85 (Fig. 4); this is in marked contrast to keratinase conjugate which exhibited minimum solubility at pH 3.6.

The absorption spectrum of keratinase dissolved in acetate buffer (pH 5.0) showed maximum absorbancy at 277 mμ; absorbancy due to nucleic acid was absent. Based on crystalline material (preparation Ta) previously subjected to analytical drying at 25° *in vacuo*, a value for $E^{1\%}_{1\,cm} = 10.42$ was calculated for absorbancy at 280 mμ.

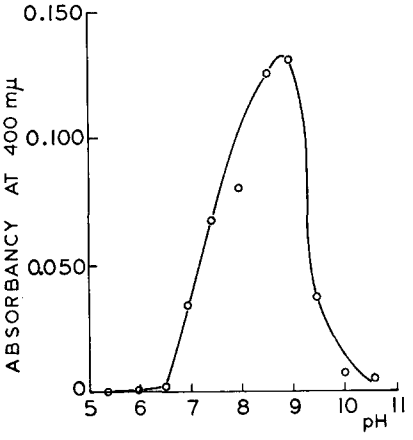

Fig. 4. Effect of pH on the solubility of crystalline keratinase. Preparation Q-S employed; details given in text.

Electrophoretic mobility of crystalline keratinase

Preparation Q-S of crystalline keratinase was dissolved in 0.05 M glycine–HCl buffer (pH 3.13) made 0.15 M in NaCl, to give a concentration of 1 mg/ml. The specific conductance of the buffer was determined to be 0.0082 ohm^{-1}/cm. Electrophoretic mobility was determined in a 2.0-ml analytical cell of a model 38A Perkin–Elmer electrophoresis apparatus equipped with a Land Polaroid camera. From photographs of the ascending boundary taken at an interval of 4140 sec, with a current of 20 mA and 100 V, a migration of 1.35 cm was observed. Employing the usual formula for the calculation of mobility, a value for $\mu = 3.99 \cdot 10^{-5}$ $cm^2/sec/V$ was obtained (Table III). As shown in Fig. 5, crystalline keratinase is electro-

TABLE III

ELECTROPHORETIC MOBILITY OF CRYSTALLINE KERATINASE AS A FUNCTION OF pH*

pH	*Buffer*	*Mobility* ($\mu \times 10^{-5}$)
3.13	Glycine–HCl	+ 3.99
5.04	Acetate	+ 1.44
6.01	Phosphate	+ 1.10
8.04	Tris	+ 0.35
9.64	Glycine–NaOH	− 0.33

* Concentration of enzyme (preparation Ta) 0.1% in 0.05 M buffer made 0.15 M in NaCl.

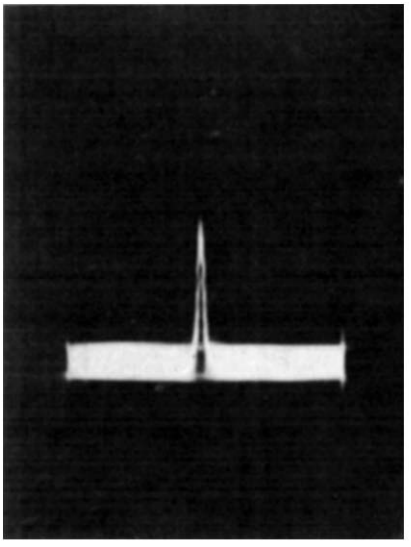

Fig. 5. Electrophoretic homogeneity of crystalline keratinase. Preparation Q-S employed with glycine–HCl buffer (pH 3.0).

phoretically homogeneous. The effect of pH at constant molarity (0.2 M) on the electrophoretic mobility of crystalline keratinase is shown in Fig. 6 wherein it can be seen that minimum mobility occurs at about pH 8.9, in excellent agreement with the data for minimum solubility shown in Fig. 4.

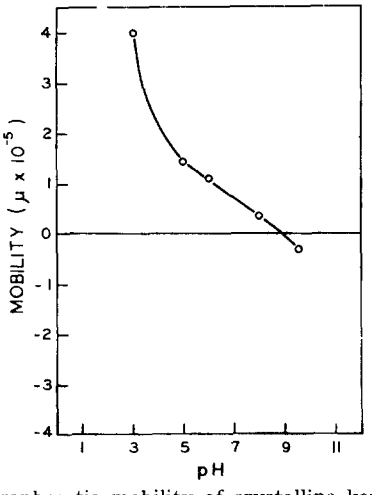

Fig. 6. Effect of pH on electrophoretic mobility of crystalline keratinase. Preparation Ta employed; details given in text and Table II.

Sedimentation of keratinase

Crystalline keratinase (preparation Ta) was dissolved in 0.2 M acetate buffer (pH 3.0) and examined in a Spinco analytical ultracentrifuge. The resulting sedimen-

tation pattern (Fig. 7) showed this crystalline material to be monodisperse; a sedimentation constant $s_{20,w}$ (uncorrected) was calculated at a concentration of 1% C to be 2.4 S. For an average protein this value would imply a mol. wt. of 27 000.

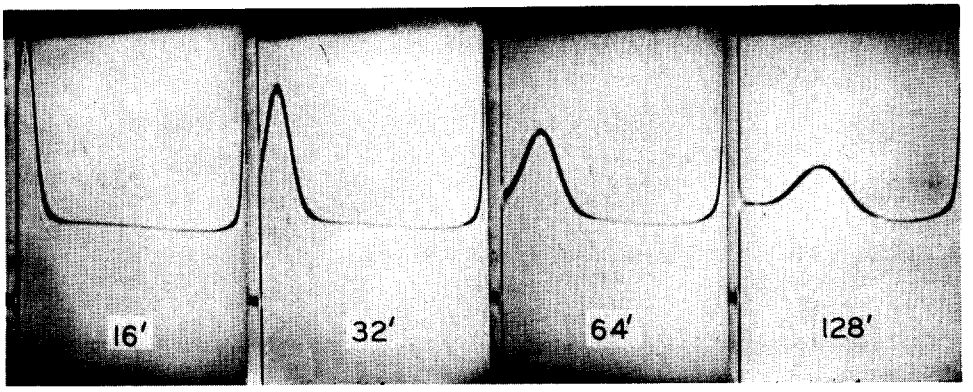

Fig. 7. Ultracentrifugal homogeneity of crystalline keratinase. Preparation Ta in 0.2 M acetate buffer (pH 3.0); duration of centrifugation noted on photographs.

Activity of keratinase on soluble substrates

Against hemoglobin substrate crystalline keratinase, on a weight basis, is about 4.7 times more active than 2-times crystallized trypsin (Fig. 8). In marked contrast to trypsin, crystalline keratinase is completely insensitive to an equal weight of soy-bean trypsin inhibitor.

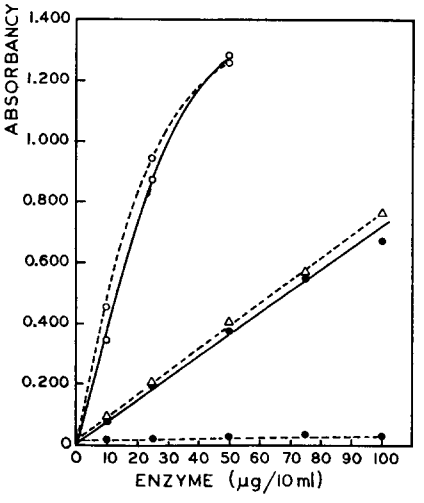

Fig. 8. Effect of soy-bean protein on activity of keratinase and trypsin against hemoglobin substrate. Crystalline keratinase, preparation Tb (○—○) same, in presence of soy-bean protein at 2 × enzyme concentration (○- - -○); trypsin alone (●—●), and in presence of soy-bean protein at 1 × enzyme concentration (●- - -●); keratinase conjugate (K4A) either alone or in presence of soy-bean protein at 1 × enzyme concentration (△- - -△).

Crystalline keratinase was incubated in Tris buffer (pH 9.0) with a variety of synthetic peptides (Table IV). The incubation mixtures were spotted on paper, chromatographed in a butanol–acetic acid–water system, and developed with ninhydrin spray. With the exception of poly-L-lysine, none of the many substrates tested gave evidence of being cleaved by keratinase. Hydrolysis of poly-L-lysine by trypsin was investigated by WALEY AND WATSON[4]; tetra-, tri-, and di-lysine were

TABLE IV

PEPTIDES NOT ATTACKED BY CRYSTALLINE KERATINASE

Dipeptides	*Tripeptides*	*Polypeptides*
L-Alanyl-L-leucine	DL-Alanylglycylglycine	Tetraglycine
DL-Alanyl-L-leucine	Glutathione	Oxidized glutathione
DL-Alanyl-L-naphthamide	Glycyl-L-leucyl-L-tyrosine	Poly-L-aspartic acid
Glycyl-L-aspartate	Glycyl-L-tyrosyl-glycinamide	Poly-glycine
Glycyl-L-glutamate		Poly-γ-benzylglutamate
Glycylglycine		Poly-L-phenylalanine
Glycyl-L-lysine		Poly-L-glutamic acid
Glycyl-L-leucine		Poly-L-benzylaspartate
Glycyl-DL-norleucine		Poly-L-tyrosine
Glycyl-L-proline		
Glycylserine		
Glycyl-L-tyrosine		
Glycyl-DL-valine		
DL-Leucylglycine		
L-Leucyl-β-naphthamide		
L-Lysylglycine		
L-Histidylhistidine		
L-Tyrosylglycine		
DL-β-Phenylserine		

demonstrated chromatographically to be hydrolytic products. On prolonged incubation ($>$ 16 h), di-lysine was the principal product; some tri-lysine was present, and only a trace of mono-lysine was apparent. Crystalline keratinase rapidly converted poly-L-lysine into four hydrolytic products (Fig. 9). In addition to tetra-, tri-, and di-lysine, a polymer with lower migration was also obtained. On prolonged incubation (24 h), no trace of mono-lysine was detected; di- and tri-lysine were present in approximately equal amount, and a substantial amount of treta-lysine remained but only faint traces of a higher polymer were evident.

Properties of the acidic component of keratinase conjugate

As described in a previous section, keratinase conjugate was cleaved on treatment with DEAE-cellulose. Dark brown, acidic material devoid of proteolytic activity was bound to the cellulose, while basic protein with undiminished keratinolytic activity was obtained in the filtrate. The bound acidic material was released from cellulose by treatment with 0.1 N NaOH. Material thus obtained in alkaline solution was purified by repeated precipitation at pH 2.5, centrifugation, and dissolution of the centrifugate at pH 7.3. Following several repetitions of this procedure, a solution of the dark material was exposed to CMS-cellulose (Brown Co.) at pH 7.3

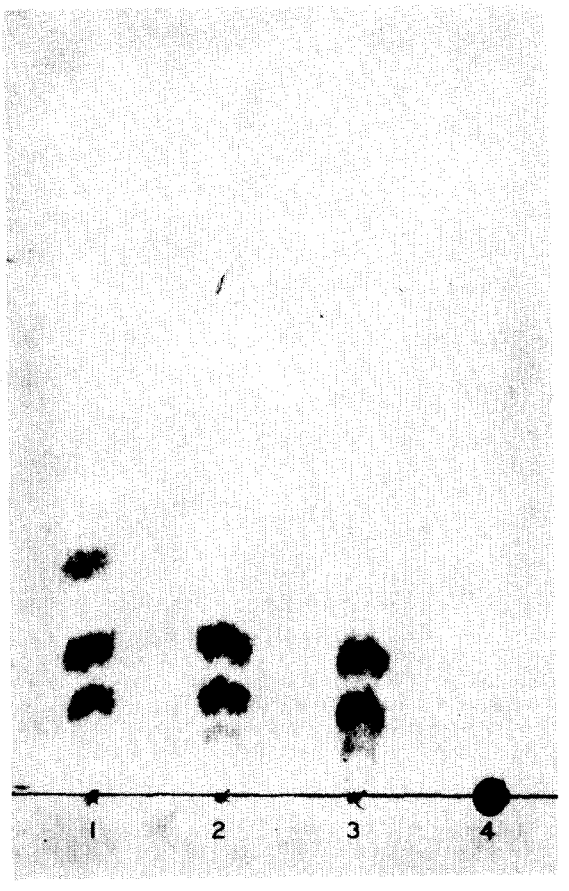

Fig. 9. Hydrolysis of poly-L-lysine by keratinase. Poly-L-lysine·HBr (5 mg) incubated for 24 h in 3 ml of 0.05 M Tris buffer (pH 9.0) at 37° with 50 μg of (1) keratinase conjugate, K4A; (2) crystalline keratinase, preparation Q-S; (3) crystalline keratinase, preparation Tb; and (4) substrate alone. Solvent system: butanol–acetic acid–water–pyridine (30:6:24:20, v/v); ninhydrin color development. In (1) the spots correspond to tri-lysine (R_F = 0.06), di-lysine (R_F = 0.09), and mono-lysine (R_F = 0.13); in (2) and (3), the slowest moving component (R_F = 0.03) corresponds to tetra-lysine; a higher polymer of (R_F = 0.015) has been hydrolyzed during the prolonged incubation.

and filtered. The dark filtrate was dialyzed overnight against running tap water. The non-dialyzable fraction was concentrated to small bulk in an Evapomix (Buchler Instrument Co.) and examined in a variety of ways.

Purified acidic material (AM) absorbed (Fig. 10) intensely in the ultraviolet. ($E^{1\%}_{1\,cm}$ at 280 mμ = 78.8) exhibiting a shoulder in the 260–280-mμ region, with end absorption rising markedly below 240 mμ (due, possibly, to light scattering). Solubility of AM in glycine–HCl buffer was examined over a pH range from 1.60 to 5.10; minimum solubility was observed at pH 2.5. The material was markedly soluble above pH 4.0. No effervescence was detected on dissolving AM in bicarbonate solution, and no evidence of phenolic characteristics was obtained with aqueous ferric

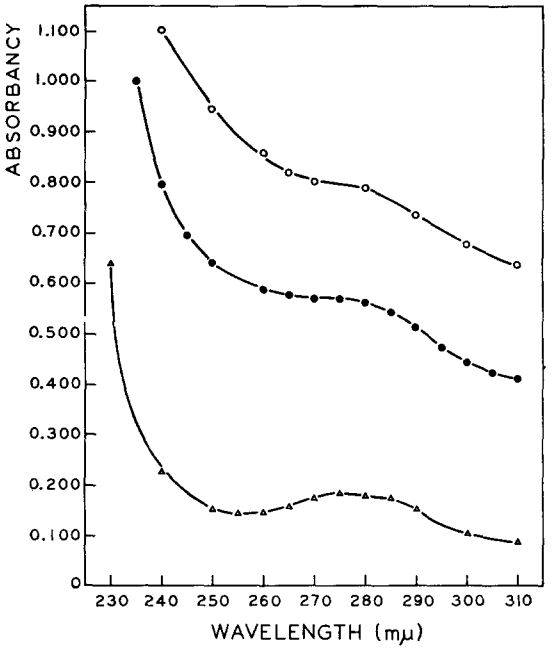

Fig. 10. Absorption spectra of purified acidic material (○—○), keratinase conjugate, K4A (●—●), and of purified keratinase lyophilized after elution from DEAE-cellulose (△—△). All substances at 100 μg/ml, in 1-cm cells. Extinction coefficients at 280 mμ (1 mg/ml): AM = 7.88; purified keratinase (free of AM) = 1.77.

chloride solution. When added to a 1% aqueous solution of protamine sulfate, a solution of AM yielded an insoluble complex with protamine.

When reacted with anthrone, AM gave a positive reaction for carbohydrate. Although the biuret test on AM was negative, the presence of several amino acids was detected after AM was hydrolyzed in 6 N HCl at 105° for 16 h. From the information presently available, AM appears to be a markedly acidic, non-dialyzable substance, devoid of nucleic acid and protein, but containing amino acids and carbohydrate. It appears to be a novel type of polymeric substance, and efforts will be directed toward elucidating its nature.

DISCUSSION

A recent review[5] of bacterial and mold proteases pointed out that several of these enzymes are known to hydrolyze both proteins and a wide variety of oligo-peptides. In fact, no microbial enzyme has yet been clearly shown to be active only on proteins. It was stated[5] that, "In contrast to animal enzymes, all well-characterized microbial proteases have very wide ranges of side-chain specificity". Of particular interest in this connection is a protease found[6] in culture filtrates of *Streptomyces griseus* grown for streptomycin production. This crystalline protease hydrolyzed 87% of the total number of peptide bonds in egg albumin, and 75% in casein, from which about twenty different free amino acids were released. The *S. griseus* protease was optimally

active between pH 7 and 8 on all substrates tested, had an isoelectric point at about pH 5.5, was strongly protected by calcium ions, and did not appear to require a reductant as activator. It catalyzed hydrolysis of an extremely wide range of synthetic dipeptides, tripeptides, acylamino acids, acyl peptides, amides, and esters[7].

By contrast, keratinase, elaborated by *Streptomyces fradiae*, is seen to be a most unusual microbial protease. Not only does it possess the unique property of attacking native keratin, its proteolytic action is directed only against proteins from which it releases peptides. Free amino acids have not been detected in filtrates resulting from incubation of keratinase with wool and, thus far, no oligopeptide (other than a decomposition product of poly-L-lysine) has been found to serve as a substrate for keratinase.

Conversion of the conjugated form of keratinase to the basic protein form by the removal of an acidic polymer resulted in surprisingly little change in catalytic properties. Despite their widely different isoelectric points, the two forms of the enzyme are similar in bringing about the digestion of $^1/_3$ the weight of native wool, in pH optimum, resistance to soy-bean trypsin inhibitor, and in releasing only peptides from wool. In view of the fact that the acidic polymer is stripped from the basic protein by an exchange reaction on DEAE-cellulose, linkage in the enzyme conjugate is probably salt-like. The linkage, however, is remarkably sturdy since the conjugate is stable over a wide pH range, and withstands both dialysis and repeated ammonium sulfate precipitation. Although the enzyme conjugate has a number of free acidic groups, the acidic polymer is evidently bound to keratinase in a manner such that the basic function which determines the pH optimum of the enzyme is free.

ACKNOWLEDGEMENTS

Supported in part by grants from the National Institutes of Health, U.S. Public Health Service. We wish to thank Dr. D. E. WILLIAMS, Research Division, Merck, Sharpe and Dohme, Rahway, N.J., for his kindness in providing the ultra-centrifugal analysis of keratinase.

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