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KERATINASE

I. PROPERTIES OF THE ENZYME CONJUGATE ELABORATED BY *STREPTOMYCES FRADIAE*

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SUMMARY

A keratinolytic enzyme secreted by *Streptomyces fradiae* is shown to have the property of solubilizing more than one-third the weight of unaltered wool. As elaborated by the organism, the protease is conjugated to an acidic polymer; this form of the enzyme is termed keratinase conjugate. In this form the enzyme exhibits optimum activity at pH 9.0 and minimum solubility at pH 3.6. Adsorption of the soluble enzyme onto wool is indicated to be a factor determining the kinetics of wool digestion by keratinase conjugate.

INTRODUCTION

A strain of *Streptomyces fradiae*, isolated from soil by an elective culture technique, rapidly digested native keratinaceous materials that had been cleansed only by mild treatment and sterilized by exposure to gaseous ethylene oxide[1]. Cell-free filtrates of cultures grown on a keratinaceous substrate possessed marked proteolytic action on wool[2]. The proteolytic enzyme secreted by *S. fradiae* has been concentrated and purified. The enzyme elaborated by this microorganism is a conjugate composed of a basic protein and an acidic polymer; properties of the protein-conjugate are described in this paper. Resolution of the conjugate into its components, and crystallization of the protein moiety are described in an accompanying paper[3].

METHODS

Microbiological methods

Isolation of a microorganism, identified as a strain of *Streptomyces fradiae*, that rapidly attacked native wool has been described[1]. In a medium containing mineral salts and native wool (as sole source of carbon and nitrogen), the organism brought about essentially complete solubilization of the wool within 8 days incubation at 28°. Wool employed in these studies was clipped from Dorset ewes, defatted in redistilled

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neutral solvents under an atmosphere of nitrogen at room temperature, and sterilized by exposure to gaseous ethylene oxide for 18 h at room temperature. It was shown that this method of sterilization did not increase the susceptibility of wool to digestion by trypsin nor alter the sulfur, nitrogen, or cystine content of wool[1].

The following basal salt formula was employed in the preparation of wool or hoof-meal media; quantities are given in g/l: K_2HPO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.025; $CaCl_2$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.015; and $ZnSO_4 \cdot 7H_2O$, 0.005. The pH of the basal salt solution was 8.3–8.4 before or after autoclaving; $CaCl_2$ was always added last to minimize precipitation.

Materials

Microcel E and Celite 345 were obtained from Johns Manville Co., New York, N.Y., and pulverized hoof-meal from the Mearl Corporation, Eastport, Maine. A standard commercial wool (Stock 64s grade), a grade routinely employed industrially to standardize batches of dyes, was obtained from Testafabrics, Inc., New York, N.Y., and was used as substrate in a wool-digestion assay. Tris, purified titrimetric standard, was obtained from Fisher Scientific Co., New York, N.Y., and employed in preparation of buffer solutions. Trypsin and papain, both 2-times crystallized preparations, and hemoglobin substrate powder were obtained from Worthington Biochemical Corp., Freehold, N.J. Folin–Ciocalteau phenol reagent was obtained from Harleco Co., Philadelphia, Pa.

Analytical methods

Total sulfur was determined by a photonephelometric method[4] after oxidation to sulfate by the micro-Carius procedure. Sulfhydryl compounds were determined by the *p*-chloromercuribenzoate method of BOYER[5] or by titration with potassium ferricyanide[6]. Cystine was determined by the *p*-chloromercuribenzoate method, after hydrolysis in 6 N HCl, followed by reduction with zinc in 0.5 N HCl. Total nitrogen was determined by a semi-micro Kjeldahl method[7], and a colorimetric ninhydrin procedure was employed for determination of amino nitrogen[8].

Two-dimensional analysis of amino acids was performed by methods outlined by STEPKA[9] employing butanol–acetic acid–water *vs.* phenol–water with 8-hydroxyquinoline. For chromatography of sulfhydryl compounds, the *N*-ethylmaleimide procedure[10] was employed.

Wool-digestion assay for keratinase activity

A wool-digestion assay was devised whereby the amount of protein solubilized during the incubation period was estimated either spectrophotometrically at 280 mμ, or by use of the Folin–Ciocalteau reagent according to LOWRY *et al.*[11]. To 200 mg of a standard commercial wool, cut with scissors into pieces about 1 mm in length, the following solutions were added: buffer, 5 ml; 10^{-3} M $MgCl_2$, 0.5 ml; enzyme solution, 1.0 ml; water to make final liquid volume 10 ml. In most of the experiments conducted with keratinase conjugate, 0.025 M borate buffer (pH 8.5) or 0.02 M Tris buffer (pH 8.6) were employed. In later experiments, 0.05 M Tris buffer (pH 9.0) was

used. The mixture was incubated at 37° without agitation for specified times, usually 3 h. At the end of the period, the mixture was filtered through cheese-cloth or fluted filter paper, and solubilized protein in the clear filtrate was estimated. For each assay the following controls were employed: (a) enzyme in buffer solution, and (b) wool in buffer solution. From A_{280} values for filtrates from the complete system (wool + enzyme + buffer) there were subtracted A_{280} values of both the wool and the enzyme controls. The corrected reading was termed the ΔA_{280} value. Plots of log ΔA_{280} values *vs.* log enzyme concentration were linear over a range equivalent to 3–40 μg/ml of a purified keratinase preparation (lot K3A), as shown in Fig. 1.

To follow the course of enzyme purification, an arbitrary unit of enzymatic activity was established, based on the data shown in Fig. 1. One unit of keratinase

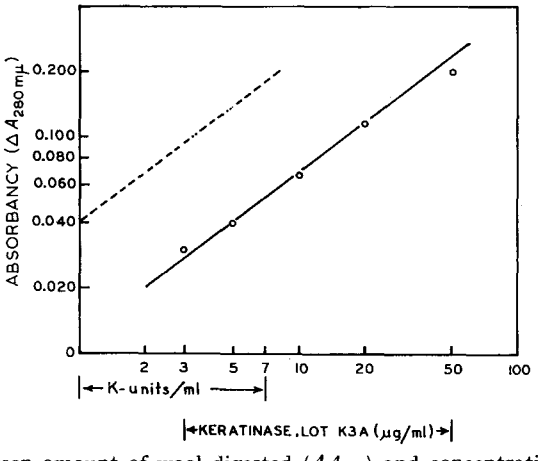

Fig. 1. Relation between amount of wool digested (ΔA_{280}) and concentration (○—○) of keratinase conjugate (preparation K3A) in standard assay procedure. Dotted line is a reference curve for expression of enzymatic activity in arbitrary K-units.

activity (K-unit) was taken as being equivalent to that obtained with 5 μg/ml of preparation K3A. To assay any sample for wool-digesting activity, it was diluted so that the increase in absorbancy, following incubation under standard conditions, fell within the range 0.040 to 0.175, corresponding to keratinase activity ranging from 1 to 7 K-units/ml (Fig. 1).

RESULTS

Elaboration of exocellular protease by S. fradiae

Removal of the filamentous growth from cultures of *S. fradiae* grown in shake flasks on a wool–salts medium or a hoof-meal–salts medium was readily accomplished by filtration *in vacuo* through Whatman No. 1 paper. The essentially cell-free filtrate was incubated in wool–buffer systems at 37° for varying lengths of time. Digestion of wool was followed by one or more methods for determining amount of wool solubilized.

For large-scale production of the wool-digesting enzyme, *S. fradiae*, strain 3739,

was grown at 28° in submerged aerated culture in 600 l of medium in a 1200-l baffled Pfaudler tank. After incubation for 44–48 h, the fermentation broth was filtered through a rotary vacuum string filter and clarified by filtration under pressure through a No. 40 Alsop asbestos filter pad. The clear filtrate was concentrated *in vacuo* at 35° to about 1/20th the original volume in a Turba-Film Evaporator. The resulting concentrates were dark brown, syrupy liquids of about 30% solids.

Keratinolytic activity of culture broths

Cell-free culture broths of *S. fradiae* possessed wool-solubilizing activity in substantial measure (Fig. 2). Activity increased with time in cultures grown on the wool medium or hoof-meal medium, reaching maximum levels after about 5 days incuba-

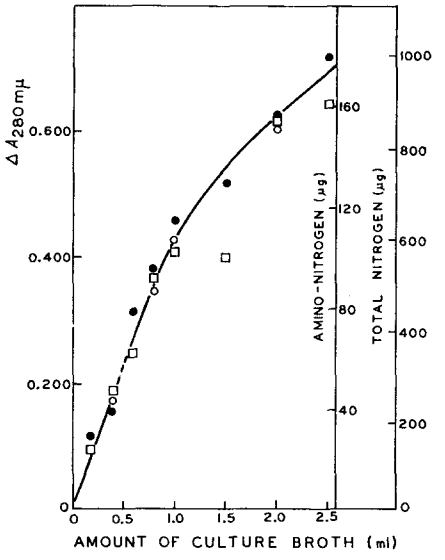

Fig. 2. Wool digestion by cell-free culture broth of *Streptomyces fradiae* strain 3739. Increase in soluble nitrogen (○—○), soluble amino-nitrogen (●—●), and material absorbing at 280 mμ (□—□), determined after incubation of 85 mg commercial wool at pH 8.6 in 10 ml of 0.02 M Tris buffer for 5 h at 37°.

tion at 28° in shake-flask cultures. Wool digestion was pronounced over a pH range from 8.0 to 9.5, and was markedly dependent upon the amount of wool supplied as substrate. With 1.0 ml of culture broth as source of enzyme, activity was dependent on substrate level up to 100 mg wool per 10 ml reaction volume. The relation between enzyme activity and amount of wool supplied as substrate will be considered further in sections dealing with the purified enzyme.

Increase in total nitrogen, amino nitrogen, and absorbancy (280 mμ) were determined in filtrates from wool-digestion assays (Fig. 2). The wool employed contained 15.8% nitrogen, and the amount of wool solubilized was calculated by multiplying total nitrogen values by 6.32. Direct proportionality between the analytical data and the amount of culture broth added (up to 1.0 ml), demonstrated that en-

zyme activity could be adequately assayed by this procedure. The scale for each ordinate in Fig. 2 was selected so that the data could be superimposed. A single line sufficed to relate the three sets of analytical values to the amount of enzyme added. In view of the heterogeneous nature of the substrate it is of interest that the products at various stages of digestion were apparently similar. From the data in Fig. 2 the ratio of increase in absorbancy (280 mμ) to the concentration of digested wool was calculated to be $A = 1.2$ (per mg solubilized products, per ml), equivalent to $E\,{}^{1\,cm}_{1\%} = 12.0$. This value, obtained from a solution at pH 8.6, is in acceptable agreement with that ($E\,{}^{1\,cm}_{1\%} = 13.5$) obtained on solubilizing this grade of wool by heating in 0.1 N NaOH.

Kinetics of wool digestion by the culture broth were examined by measuring the increase in absorbancy (280 mμ) at intervals during the incubation period. The rate of solubilization of material absorbing at 280 mμ was essentially constant for the first 5 h. On the basis of these data, a 3-h incubation time was subsequently employed in a standard assay procedure.

Enzyme purification

Two general procedures were employed for purification of the *S. fradiae* protease. Concentrated, dialysed fermentation broths were subjected to: (a) precipitation in the cold with ammonium sulfate, or (b) adsorption onto Microcel E and elution therefrom, followed by precipitation with ammonium sulfate. Concentrated, dialyzed fermentation broths were treated with ammonium sulfate at 5°. At 0.6 saturation a fine precipitate appeared which could be recovered in a Sharples centrifuge. The precipitate was lyophilized and found to contain about 3/4th of the total keratinolytic activity of the dialyzed concentrate. The first preparation thus obtained was termed lot K3A, and assayed 200 K-units/mg dry weight.

By the second procedure, a lyophilized powder was obtained that assayed 475 K-units/mg dry weight (lot K4A). Cooled, filtered fermentation broth that assayed 400 K-units/ml was adjusted to pH 6.0, and 2.0% (w/v) Microcel E was added. The mixture was agitated for 30 min, the pH being maintained at 6.0; 1.0% (w/v) Celite 545 was added and the slurry was filtered through a horizontal plate-and-frame filter press. The filter cake was washed with water until colorless, then blown dry, and removed from the press. The cake was extracted with a solution containing NaCl (1.5%) and Na_3BO_4 (0.5%). The slurry was adjusted to pH 9.0 with NaOH and agitated for 30 min. The slurry was filtered as before; filtrate and water wash of the filter cake were combined and adjusted to pH 7 with HCl. Ammonium sulfate was added to the cooled (5°) eluate to give 0.6 saturation, and let stand at 5° for 16 h. A fine precipitate developed but this was not readily separated in a Sharples centrifuge. Filtration was resorted to for recovery of the precipitate. Celite 545 (0.1%) was added with gentle agitation to the precipitated enzyme suspension, and the resulting slurry was put through a pressure filter, the filter pad of which had been precoated with Celite 545. The filter cake was suspended in cold 0.025 M Na_3BO_4 solution and agitated for 30 min. Filtrate and water washings were pooled, adjusted to pH 7.0, and lyophilized to yield a light tan powder that constituted a 40-fold purification (on a weight basis) of the activity in the initial fermentation broth. This preparation possessed proteolytic activity equivalent to that of an equal weight of crystalline trypsin when tested against hemoglobin substrate (see Fig. 6).

Properties of keratinase conjugate

The ultraviolet-absorption spectrum of purified keratinase showed a broad plateau between 260 and 285 mμ; the high absorbancy is noteworthy (Fig. 3). The extinction coefficient at 280 mμ (1 mg/ml, 1.00-cm cell) for this preparation was 5.75; a value of 5.87 was obtained with another preparation. These values are much higher than those usually obtained with proteins (0.5–1.5) and, taken in conjunction with the absorption spectrum, indicate that these preparations contain some highly absorbing, non-dialyzable material other than protein or nucleic acid. As shown in

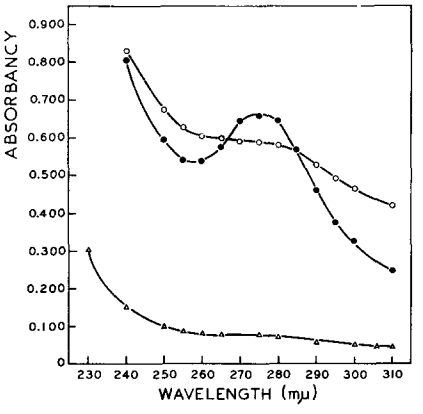

Fig. 3. Absorption spectrum of keratinase conjugate, preparation K5A, 100 μg/ml (○—○); concentrate of wool digest (200 μg/ml) in Tris buffer (pH 9) (●—●); and eluate from wool exposed to 0.05 M Tris buffer (pH 9) for 12 h (△—△).

detail in the following paper[3], this material proved to be a highly acidic polymer (which has not, as yet, been identified with any known type of polymeric substance), devoid of proteolytic activity, but to which a basic protein (keratinase) is firmly bound. The term "keratinase conjugate" will be applied to the enzyme elaborated by *Streptomyces fradiae*, and the term keratinase will be reserved for the basic protein resolved from the conjugated form.

Keratinase conjugate exerted maximum proteolytic activity against a wool substrate at pH 9.0. The increase in activity between pH 7 and 9 was abrupt; activity at pH 8.0 being slightly less than half that observed at pH 9.0. Beyond pH 9.0 activity dropped precipitously and the enzyme was rapidly inactivated. Enzymatic activity increased with rising temperature, an optimum being exhibited near 50° by both a partially purified enzyme preparation (K3A), and unconcentrated filtrate obtained from a fermentation broth. At 37°, employed for all other assays reported in this paper, the enzyme exhibited about 42% of the activity shown at 50°.

To determine the effect of pH on solubility of keratinase conjugate, buffers covering the pH range from 1.0 to 10.0 were prepared; KCl–HCl buffer was employed from pH 1.0 to 2.0, acetate buffer from pH 2.55 to 5.60, and veronal buffer from pH 3.5 to 9.50. To a tube containing 4.0 ml buffer, 0.2 ml of a 1% (w/v) solution of a purified enzyme preparation (K4A) was added. The tubes were let stand for 18 h at 4°; supernatant fluid in each tube was carefully decanted, then centrifuged

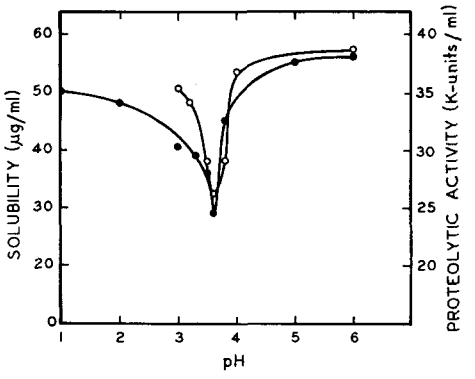

Fig. 4. Effect of pH on solubility of keratinase conjugate. Preparation K4A employed in experiment described in text; solubility expressed as μg solute/ml (●—●) and as enzymatic activity in K-units/ml (○—○).

at 10 000 × *g*. The clear supernatant solutions were assayed for protein content and keratinase activity. As shown in Fig. 4, keratinase conjugate is a markedly acidic material with minimum solubility at pH 3.6.

Enzymatic activity against insoluble substrates

The amount of wool solubilized in a given time by a given concentration of enzyme increased rapidly with increasing amounts of wool up to about 200 mg wool per 10 ml reaction volume, and gradually leveled off above that amount. In our standard assay procedure, 200 mg of wool per 10 ml reaction volume was routinely employed. The wool substrate is, of course, essentially insoluble in pH 9.0 buffer. That such a relatively vast amount of an insoluble substrate should, nevertheless, be rate-limiting implies limitation in surface for adsorption of enzyme onto wool.

A log–log plot of amount of substrate *vs.* ΔA_{280} showed a linear relationship over a range from 20 to 400 mg wool per 10 ml reaction volume (Fig. 5). Proteolytic

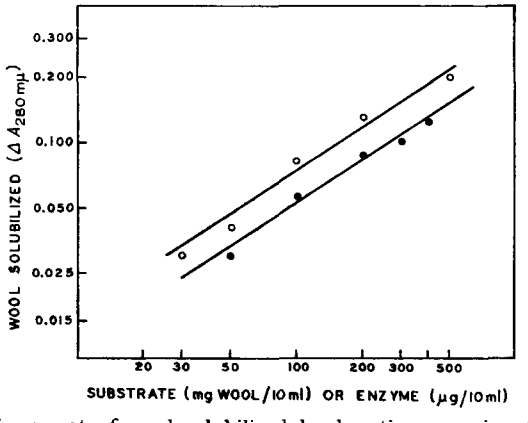

Fig. 5. Dependence of amount of wool solubilized by keratinase conjugate on the amount of substrate supplied (●—●) and on enzyme concentration (○—○). Preparation K3A employed in standard assay procedure with 200 mg wool (enzyme concentration varied), or 20 μg/ml of enzyme (wool varied); logarithmic plot.

activity of keratinase conjugate against wool substrate was also linearly proportional to enzyme concentration over a range from 3 to 40 μg/ml when expressed in the form of a log–log plot (ΔA_{280} *vs.* enzyme concentration, Figs. 1 and 5). This relationship suggests that adsorption of soluble enzyme onto insoluble wool substrate is a major factor in the kinetics of wool digestion by the enzyme.

The empirical isotherm proposed by FREUNDLICH[12] to describe adsorption from solution onto the surface of a solid adsorbent is frequently expressed in the form:

$$y = kc^n \tag{1}$$

and a plot log y *vs.* log c is linear with slope n. In the equilibrium relationship expressed in Eqn. 1, y represents weight of substance adsorbed per unit weight of adsorbent, c is the equilibrium concentration of adsorbed substance, while k and n are constants that depend on temperature.

The data shown in Fig. 5 may be expressed in a similar manner:

$$\log w = n \log W + \log k \quad (E \text{ fixed}) \tag{2}$$

$$\log w = n' \log E + \log k_1 \quad (W \text{ fixed}) \tag{3}$$

where w is a measure of the amount of wool digested, W the amount of wool supplied, and E the amount of enzyme supplied; n and n' are constants given by the slopes of the lines, while log k and log k_1 are constants given by the y intercepts in Fig. 5.

Rewriting Eqns. 2 and 3 we obtain:

$$w = k\,(W)^n \tag{4}$$

$$w = k_1\,(E)^{n'} \tag{5}$$

The slopes n and n' are essentially indistinguishable (0.52 and 0.53, respectively); Eqns. 4 and 5 thus indicate that the amount of wool solubilized varies essentially as the square root of the enzyme or wool concentrations in the range of values tested.

Activity of keratinase conjugate on soluble proteins

In addition to its property of solubilizing substantial portions of keratinaceous materials such as wool and feathers, keratinase rapidly digest casein, azocasein (prepared by the procedure of CONKLIN[13]), and denatured hemoglobin substrate (procedure of ANSON[14]). Activity of keratinase against the latter substrate is shown in Fig. 6 wherein it will be noticed that arithmetic scales are employed. A linear

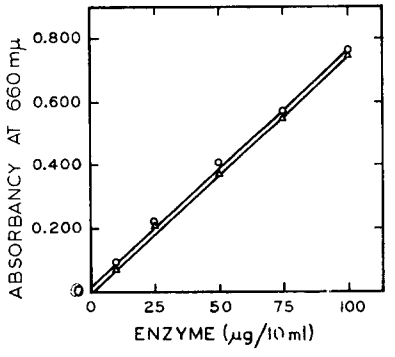

Fig. 6. Activity of keratinase conjugate (○—○) and of crystalline trypsin (△—△) against hemoglobin substrate at pH 7.5 (assay procedure of ANSON[14]).

relationship between enzyme concentration and enzyme activity has been observed with the three soluble substrates tested (casein, azocasein, and denatured hemoglobin). This observation is in contrast with that evident with insoluble substrates (see Fig. 5), and serves to emphasize that adsorption processes govern the action of keratinase on wool.

Comparison of action of trypsin and keratinase on wool

Solutions of crystalline trypsin and of keratinase conjugate (preparation K4A) were made in appropriate buffers to give final concentrations of 250 μg/ml. Tris buffer, 0.05 M, adjusted to pH 8.0, was employed for trypsin, and to pH 9.0 for keratinase. For each enzyme the following set of flasks was prepared: (a) wool + enzyme + buffer, (b) wool + buffer, (c) enzyme + buffer. Total liquid volume in each flask was 25 ml and 170 mg dry wt. of wool was contacted with 6.25 mg of enzyme. Toluene was added to each flask to maintain sterility during the long incubation period. All flasks were incubated without agitation at 37° for periods stated in Table I, at the end of which times the contents of each flask were filtered through

TABLE I

COMPARISON OF ACTION OF TRYPSIN AND KERATINASE CONJUGATE ON WOOL*

Incubation time (h)	A_{280}	*Protein solubilized*		
		260/280 mμ		*Ninhydrin***
		mg	*Per cent of total*	*mg*
		Keratinase conjugate		
16	1.34	31.9	18.7	8.01
20	1.57	37.1	21.8	9.67
37.5	1.97	48.6	28.6	13.92
56.5	2.18	53.9	31.7	16.85
126.5	2.63	62.4	36.7	—
		Trypsin		
16	0.083	1.8	1.0	0
20	0.097	2.0	1.2	0
37.5	0.190	5.5	3.2	0
56.5	0.258	7.7	4.5	0

* Values given on a cumulative basis.
** Values expressed with reference to leucine standard.

Whatman No. 1 paper, and absorbancy of the clear filtrates was determined at 260 and 280 mμ. The ΔA values thus obtained were converted into mg protein/ml of solution by calculation according to the method of KUNITZ[15]. After exposure to four applications of keratinase, the wool had lost 31.7% of its weight. In the same time, wool exposed four times to trypsin lost only 4.5% of its weight. Included in Table I are values for weight of amino nitrogen solubilized.

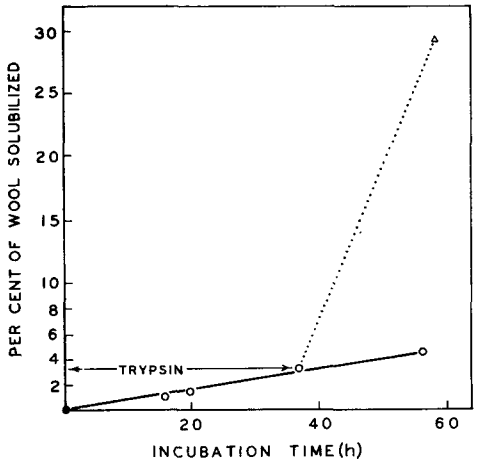

Fig. 7. Activity of keratinase conjugate (○.....△) on wool that had been exposed three times to trypsin (○—○). Details given in text.

Samples of wool that had been exposed over a period of 37.5 h to 3 consecutive treatments with crystalline trypsin (under conditions as stated above) were separated by filtration, washed with water, then exposed to fresh trypsin solution at pH 8, or to keratinase conjugate (K4A) at pH 9. Incubation, in the presence of toluene, was continued for 19 h at 37°. At the end of this time the contents of the flasks were filtered, and the amount of wool solubilized was determined; the results are compared in Fig. 7. The effectiveness of keratinase in solubilizing a very substantial amount of wool that had been exposed repeatedly to trypsin is evident.

The data given in Table I for digestion of wool by keratinase conjugate were calculated as weight of wool solubilized per hour of incubation. As seen from Fig. 8,

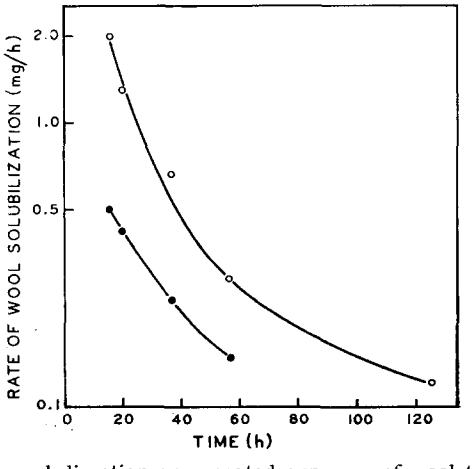

Fig. 8. Decline in rate of wool digestion on repeated exposure of wool to keratinase conjugate. Digestion products estimated spectrophotometrically (○—○) or by determination of amino nitrogen (●—●).

the rate of wool solubilization declines progressively in a manner such that an asymptote is approached when slightly more than one-third of the weight of wool has been digested. After the first exposure to keratinase conjugate only 25% of the weight of peptides released from wool are detected by ninhydrin analysis whereas, after the fourth exposure to enzyme, smaller peptide moieties are solubilized since the ninhydrin analysis detects more than 50% of the total released.

Sensitivity of trypsin and keratinase conjugate to soy-bean trypsin inhibitor was compared on denatured hemoglobin substrate. Complete inhibition of trypsin was achieved at equal concentrations by weight of enzyme and inhibitor. In contrast, an equal weight of the soy-bean protein depressed activity of keratinase conjugate by only 5%, and a 20-fold excess weight caused only 40% inhibition.

Products of action of keratinase on wool

During growth of *S. fradiae* in a wool–salts medium, three sulfhydryl-containing

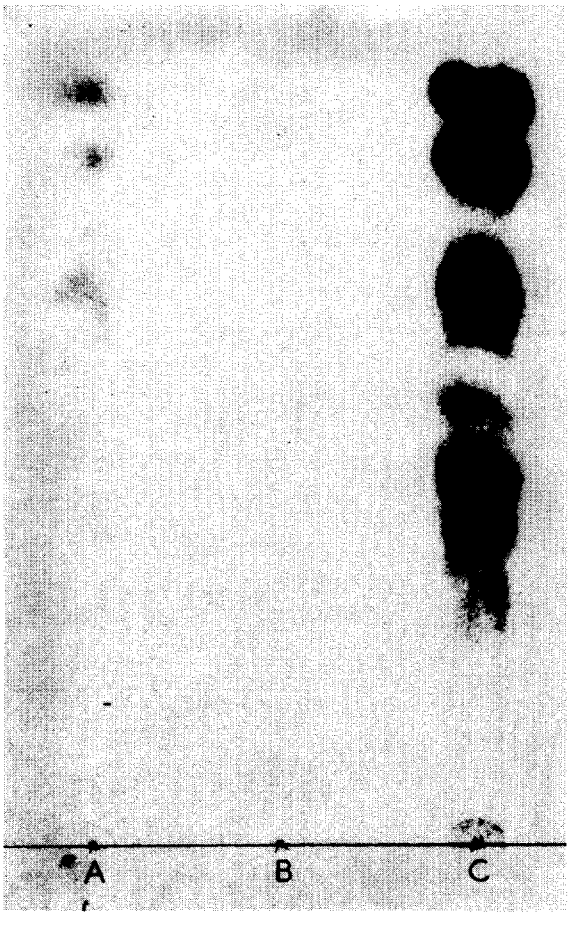

Fig. 9. Chromatogram of ninhydrin-positive substances solubilized from wool by the action of keratinase conjugate; A, enzyme alone; B, wool alone; C, wool + enzyme.

substances were detected in the fermentation broth. Chromatography of the *N*-ethylmaleimide derivatives of these substances showed them to be distinct from cysteine and glutathione[16].

In contrast to the action of the organism on keratinaceous materials, the enzyme elaborated by *S. fradiae* does not cause release of sulfhydryl-containing peptides from wool. Water-soluble ninhydrin-positive products resulting from digestion of wool by keratinase conjugate were resolved by paper chromatography, as shown in Fig. 9. On elution from paper and subjection to acid hydrolysis, areas corresponding to the spots shown in Fig. 9 were found to be comprised of peptides of varying sizes. This work is being extended in an effort to characterize amino acid sequences in individual peptides that have been isolated, and will subsequently be reported in detail. Since the enzyme described in this paper has the property of solubilizing more than one-third the weight of essentially undenatured wool, and the digestion products are peptides of varying size, the prospect of unravelling some of the amino acid sequences of a keratinaceous material emerges.

DISCUSSION

Native keratinaceous structures such as horn, wool, or feathers are almost completely resistant to attack by proteolytic enzymes hitherto known. For example, GEIGER *et al.*[17] reported that crystalline trypsin, pepsin, papain, or chymotrypsin in 0.1% solutions caused no loss in tensile strength of wool which had received no treatment other than cleansing with alcohol, ether, and water at room temperature. On the other hand, wool that had been reduced with alkaline thioglycollate (*i.e.* "kerateine") was readily digested by these enzymes[17,18]. Oxidation of reduced wool preparations, resulting in reformation of disulfide bonds, did not prevent digestion of the wool by trypsin. It should be noted that the reoxidized "wool" was amorphous and did not yield a distinct X-ray pattern. ROUTH[19] found that finely chopped or whole fibers of wool were very slowly attacked by trypsin, whereas wool that had been powdered by ball-miling was digested by trypsin at a rate equal to 50% of that at which casein is digested by trypsin. In general, most treatments which chemically or mechanically affect wool, also make a greater percentage of it susceptible to enzymatic digestion[20].

The term "keratinase" can be found in the literature (*e.g.* SUMNER AND SOMERS[21]), and has been used to indicate an entity the existence of which might be anticipated. In the investigations of LINDERSTROM-LANG AND DUSPIVA[22] on digestion of wool by clothes-moth larvae, the midgut portion (in which wool digestion is effected) was shown to be characterized by a low redox potential, and an alkaline pH. A tryptic type of proteolytic enzyme was presumed to digest the altered keratin (kerateine) obtained after non-enzymatic reduction of some of the many -S–S-linkages of keratin. *In vitro*, digestion of unaltered wool by the clothes-moth protease was negligible.

Three enzymes are known so far to catalyze cleavage of disulfide bonds; all necessitate participation of some metabolically generated hydrogen donor—cystine reductase[23,24] operates most commonly with DPNH; glutathione reductase[25,26] with TPNH; and protein disulfide reductase[27] *via* a complex system involving reduced pyridine nucleotide[28] and, presumably, a reduced flavin[29]. Each of these enzymes requires the operation of systems regenerating its respective hydrogen donor. All

three of these enzymes appear to be widely distributed in nature; of especial interest, POWNING AND IRZYKIEWICZ[30] found cystine reductase to be very active in clothes-moth larvae. The "diffusible reducing agent," implicated by LINDERSTROM-LANG AND DUSPIVA[22] in digestion of wool by clothes-moth larvae, would appear to be cysteine.

Eleboration of an exocellular proteolytic enzyme capable of causing substantial digestion of native wool is, apparently, a unique attribute of strains of *Streptomyces fradiae*. The enzyme (keratinase) accomplishes degradation of keratin in some manner other than by cleavage of disulfide bonds. No dialyzable cofactor is essential for activity of keratinase, nor is any reducing activator (as with papain) required. Furthermore, keratinase is essentially insensitive to substances, such as soy-bean protein, that inhibit trypsin. Although keratinase conjugate has not as yet been obtained in crystalline form, purified preparations of it possess proteolytic activity equivalent to that of an equal weight of crystalline trypsin when tested against denatured hemoglobin, a substrate attacked both by trypsin and keratinase conjugate. Since the best preparations of keratinase conjugate possessed about 1/10th the specific activity (K-units/mg dry wt.) of crystalline keratinase[3], several lines of evidence were adduced to show that keratinase conjugate is, in fact, a "conjugate" and not merely an impure preparation with 90% "contamination". The material termed keratinase conjugate was shown to have the following properties: (a) be adsorbed onto Microcel E (a diatomaceous material) and be eluted therefrom by alkaline solutions, (b) be precipitated with ammonium sulfate at 0.6 saturation, (c) exhibit minimum solubility at pH 3.6, and (d) be cleaved[3] by ion-exchange cellulose into an acidic polymer (retained on column) and a basic protein (obtained in effluent). Since keratinase activity was a property of the basic protein (which exhibits minimum solubility[3] at pH 9.0), it is clear that the protein portion of keratinase conjugate must be bound to the acidic polymer *via* relatively stable salt linkages.

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