BBA 65820

ISOLATION AND PROPERTIES OF AN EXTRACELLULAR PROTEASE OF *TRICHOPHYTON GRANULOSUM*

WILLIAM C. DAY*, PAULINE TONCIC, SANDRA L. STRATMAN, URSULA LEEMAN AND SANDRA R. HARMON

The Skin and Cancer Hospital, Temple University Health Sciences Center, Philadelphia, Pa. (U.S.A.)

(Received June 5th, 1968)

SUMMARY

1. An extracellular protease of the dermatophyte *Trichophyton granulosum*, used in the initial digestion of hair substrates, has been isolated from the culture filtrate. The procedure for cultivating the fungus on horse hair is given.

2. A method is described for the purification of the protease by $(NH_4)_2SO_4$ fractionation and chromatography on CM-cellulose and Sephadex G-100.

3. The protease is activated by alkaline earth ions, whereas other bivalent cations are generally inhibitory.

4. Caseinolytic activity was maximal at pH 9.5–9.8, 45° and $I = 0.10$.

5. The enzymatic activity was rapidly destroyed below pH 4.7 and above pH 8.0, and stability maxima were indicated at these two values.

6. An amino acid analysis indicates a large tryptophan content. This correlates with the $E^{1\%}_{1cm}$ value of 22.15.

7. A molecular weight of 34 300 was estimated for the enzyme by gel filtration.

8. A series of peptides was not hydrolyzed by the enzyme. The A and B chains of oxidized insulin were degraded but not to the amino acids.

INTRODUCTION

The dermatophytes are parasitic fungi causing disease (ringworm) in man and animals. Their main pathogenic feature is their ability to invade and thrive in the keratinized layers of skin, hair and nails. This peculiar physiological property, to utilize keratin as the sole source of nitrogen, has attracted interest ever since pure cultures of dermatophytes were first isolated.

The enzymatic properties of dermatophytes were observed by MACFADYEN[1] and ROBERTS[2,3], but it was BODIN[4] who first demonstrated the proteolytic activity of

* Present address: Biochemistry Department, University of Alberta, Edmonton, Alberta, Canada.

Microsporum gypseum. The proteolytic enzymes of fungi of the Trichophyton group have been studied by CRUICKSHANK AND TROTTER[5] and CHATTAWAY, ELLIS AND BARLOW[6]. However, to our knowledge, the isolation of the extracellular protease responsible for the keratinolytic activity of a dermatophyte, has never been reported. This paper reports the preparation, isolation and some properties of an extracellular protease of *Trichophyton granulosum*. The data reported herein indicate that this protease is primarily responsible for the ability of *T. granulosum* to utilize hair.

MATERIALS AND METHODS

Materials

All chemicals unless otherwise specified were Baker Analytical Reagent or Merck Reagent Grade purchased from A. H. Thomas. Amino acids, peptides and proteins were M.A. products from Mann. Sephadex G-100 and Blue Dextran were purchased from Pharmacia and ion-exchange celluloses were from Reeve Angel.

Guinea pig hair used as substrate was sheared from white adult animals, Hartley strain, of our colony. Horse hair was obtained from Samuel Zeitlin's Sons, Inc., Philadelphia.

Trichophyton granulosum Sabouraud, 1909, Strain No. 4, cultivated for the enzyme production, had been isolated from a white laboratory rat.

Sörenson's phosphate buffer was used in the experimental work, if not otherwise specified. The concentration was 0.028 M to conform with the optimal ionic strength for the activity of the protease. Inasmuch as preliminary work indicated the need of Mg^{2+} for activity, enzyme solutions were routinely maintained at 1 mM $MgSO_4$ to secure maximum proteolytic activity.

Enzyme assay

Routine measurements of proteolytic activity were performed by the digestion of casein according to a modified method of KUNITZ[7]. 1 ml of 1% casein solution in 0.028 M phosphate buffer (pH 7.8), 1 mM $MgSO_4$, was added to the enzyme sample previously diluted to 1 ml with phosphate buffer. After incubating for 20 min at 37°, the reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (w/v). The precipitate was removed by centrifugation and filtration, and the absorbance of the filtrate was measured at 280 mμ. In control tubes, the reaction mixture was acidified before the addition of casein. For assays at pH values other than 7.8, appropriate buffers were used (see Fig. 2).

Digestion of hemoglobin served for the assay of proteolytic activity of solutions at pH values below 6.0 (ref. 8). Solutions of approx. 2% alkaline urea-denatured hemoglobin solution were incubated for 10 min at 37° with a sample diluted to 1 ml with the corresponding buffer. The reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid (w/v). After centrifugation and filtration, the absorbance of the filtrate was measured at 280 mμ. Corresponding controls were run in each case.

One unit of proteolytic activity is the amount of enzyme whose reaction filtrate gave an absorbance of 0.100 at 280 mμ under the conditions of the standard caseinolytic test. The specific activity is the number of units of activity per mg of protein.

Keratin substrate preparation

Guinea pig hair, 10–15 mm long, was washed free of extraneous matter with Tween 80 solution, rinsed successively with tap and distilled water, and air-dried. Horse hair, obtained as bundles of tail hair, was cut into lengths of 3–5 mm with electric clippers. The hair was defatted following a modified procedure of BLIGH AND DYER[9]. 6 g of hair was shaken vigorously in a mixture of 39 ml of distilled water, 50 ml of chloroform and 100 ml of methanol for 15 min. 50 ml of chloroform was added and the shaking was continued 5 min. Finally after adding 50 ml of water and shaking 5 min, the hair was collected on a filter, rinsed with 50 ml of methanol–water (3:1, by vol.) and air-dried.

T. granulosum cultures

Slopes of Sabouraud's conservation agar (3% Difco neopeptone, 1% agar) were inoculated with *T. granulosum*, incubated 4 days at 23–25°, and then 8–10 weeks at 15°.

The liquid culture medium for the cultivation of *T. granulosum* consisted of the following per l of solution: 2.5 g of hair; 0.9 g of glucose; 0.6 g of $MgSO_4 \cdot 7\ H_2O$; 6.87 g of $Na_2HPO_4 \cdot 7\ H_2O$; 0.333 g of KH_2PO_4; 0.01 g of thiamine; 0.01 g of pyridoxine; and 0.05 g of inositol.

A spore suspension scraped from a slant tube was used to inoculate two 500-ml flasks containing guinea pig hair in 200 ml of culture medium. The cultures were kept 5 days in the dark and then shaken at approx. 90 rev./min on a gyrorotatory shaker. This growth, in turn, was used to inoculate 8 similar flasks which were subsequently incubated in the same fashion.

Enzyme preparation

6-l erlenmeyer flasks containing horse hair in 4500 ml of medium were each inoculated with 200 ml of fungal growth. The cultures were kept at 20–23° in the dark for 5 days and then agitated 7–9 days on the shaker. The culture fluid was separated from the mycelium and undigested hair by filtration through a coarse glass filter covered with a 1-cm layer of clean sand. Filtrates were kept at 4° and extracted with ion-exchange cellulose the following day, or frozen if not extracted within 48 h.

Enzyme concentration

Pre-treated DEAE-cellulose (1 g/60 ml of culture filtrate) equilibrated with 0.028 M phosphate buffer (pH 7.8) was stirred with culture filtrate 1 h. The DEAE-cellulose was collected on a filter and the adsorbed material was eluted with 0.5 M NaCl–phosphate buffer of approx. 5% of the original volume. The pH of the filtrate was lowered to 6.5 by the addition of 0.5 M HCl. Pre-treated CM-cellulose (1 g/60 ml) was added, the suspension was stirred 1 h and then filtered. The adsorbed protease was eluted with 0.5 M NaCl–phosphate buffer in 5% volume of the filtrate, dialyzed free of NaCl, lyophilized and stored at − 10°.

Enzyme purification

All operations were conducted at 4°.

Extracting from enzyme concentrate. The enzyme concentrate collected from 357 l of *T. granulosum* culture filtrate by CM-cellulose extraction was suspended in

200 ml of distilled water and dialyzed overnight against 0.028 M phosphate buffer (pH 7.8). Undissolved salts were removed by centrifugation and washed 3 times with 100 ml portions of phosphate buffer. The supernatants were combined, dialyzed against 0.028 M phosphate buffer and lyophilized.

Fractionation with $(NH_4)_2SO_4$. The lyophilized powder was resuspended in 200 ml of water, centrifuged to clear of insoluble material, and the 191 ml of supernatant was stirred with 127 ml of buffer saturated with $(NH_4)_2SO_4$ giving a 40% saturation with respect to the ammonium salt. After 1 h, the precipitate was removed by centrifugation (30 min, 34 000 × *g*) and discarded. The supernatant was brought to 70% saturation by the addition of 320 ml of $(NH_4)_2SO_4$ saturated buffer, and after 1 h, the precipitate was collected by centrifuging. Attempts to purify the protease by additional precipitation procedures with $(NH_4)_2SO_4$ were unsuccessful.

CM-cellulose chromatography. The enzyme sample in 10 ml of buffer was applied to a column (1.5 cm × 30 cm) of CM-cellulose (Whatman CM 52, microgranular) equilibrated with 0.05 M phosphate buffer (pH 6.0) and 1 mM $MgSO_4$. Gradient elution of the active portion was accomplished with a 9-chamber Varigrad. All chambers contained 111 ml of buffer; the buffer in chamber 3 was 0.1 M NaCl; in chamber 7, 0.4 M NaCl; and in chambers 5 and 9, 0.5 M NaCl. The proteolytic activity was eluted in a single peak at 0.15 M NaCl. The pooled fraction of the peak was divided into 4 parts, each was dialyzed against 0.05 M sodium acetate and lyophilized.

Sephadex G-100 filtration. Each of the 4 aliquots was dissolved in 1 ml of 0.1% Blue Dextran solution and layered on a Sephadex G-100 column (1.5 cm × 88 cm) prepared in 0.05 M sodium acetate buffer (pH 6.0) and 1 mM $MgSO_4$. 1-ml fractions were collected at a flow rate of 20 ml per h. The active fractions were pooled, dialyzed against 0.028 M phosphate buffer (pH 7.8), lyophilized and stored at −10°. The combined yield of purified enzyme was 8.8 mg, as determined by a direct weight differential of the lyophilized powders of sample and buffer.

The purified protease exhibited a single band in disc electrophoresis run at pH 4.3 on 7.5% polyacrylamide gel. The protease did not migrate on the standard gel.

Molecular weight determination

The molecular weight was estimated according to the method of WHITAKER[10] and ANDREWS[11] by comparing the rates of elution of the protease and of reference proteins from a Sephadex G-100 column (1.5 cm × 88 cm). 5 mg of a reference protein (ribonuclease, chymotrypsin, trypsin, pepsin, ovalbumin and γ-globulin) dissolved in 1 ml of 0.1% solution of Blue Dextran 2000 in 0.05 M sodium acetate (pH 6.0), 0.1 M NaCl, was applied to the column and the eluent was collected in 1 ml fractions and monitored at 280 mμ. The maximum of the elution peak of the Blue Dextran and of the protein peaks were approximated to one-tenth of a ml. 1-ml samples of the protease (approx. 2 mg) were similarly chromatographed.

Spectrophotometric estimation of tryptophan

The molar ratio of tryptophan to tyrosine was determined by the method of BENCZE AND SCHMID[12]. The absorption maximum of ionized tyrosine in 0.1 M NaOH solution is at 293 mμ. The slope of a line drawn tangent to maxima at 274 mμ and 293 mμ is indicative of the tryptophan to tyrosine ratio. Since the tyrosine content

was available by the amino acid determination, the tryptophan value could be determined spectroscopically.

RESULTS

Preparation, extraction and purification

The cultivation of *T. granulosum* on horse hair in a submerged culture (see MATERIALS AND METHODS) produced a filtrate high in proteolytic activity. Growth on this substrate yielded 5 protease units per ml representing 1–1.5 mg of enzyme per l of culture filtrate.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR *T. granulosum* PROTEASE

Fractionation step	*Vol. (ml)*	*Total activity (units × 10^3)*	*Specific activity (units/mg protein)*	*Recovery (%)*
Culture filtrate	357 000	2301	—	100
CM-cellulose extract	18 580	1270	—	55
Lyophilized, stored, redissolved and dialyzed	191	869	—	38
40–70% $(NH_4)_2SO_4$, 3 precipitations	15	237	—	10
Pooled CM-cellulose fractions	206	179	1633	7
Homogeneous fraction from Sephadex G-100	116	35	3990	2

The extraction and purification procedure is summarized in Table I. The small amount of protease available in the culture filtrate necessitated extracting with CM-cellulose, lyophilizing and storing in order to accumulate adequate amounts of enzyme concentrate. This concentration step involved a relatively large loss of activity.

At 40% $(NH_4)_2SO_4$, a non-active fraction precipitated and was discarded. Increasing the concentration in the supernatant to 70% yielded a precipitate which was 41% enzyme. Further attempts to improve the purity of the protease by this procedure were unsuccessful.

Of three attempts to isolate an active sample of the protease which was homogeneous on disc electrophoresis, only the reported sequence was successful. The recovery of protease was decreased by excluding overlapping portions of the active peak with other proteins in chromatography steps in order that an electrophoretically pure sample could be collected.

pH stability

The caseinolytic activity of the culture filtrate was rapidly destroyed below pH 4.7 and above pH 8.0 with stability maxima indicated at these two values (Fig. 1).

Optimum pH

The optimum pH for the caseinolytic activity of the protease was between 9.5 and 9.8. This optimum coincided with that of the culture fluid (Fig. 2).

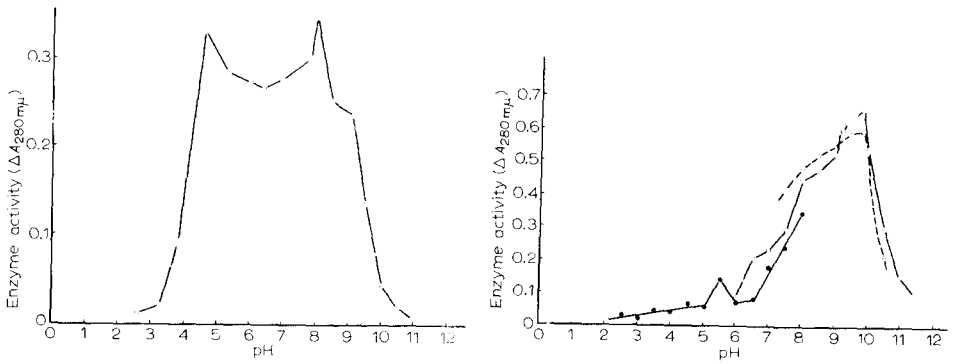


Fig. 1. The pH stability of the proteolytic activity of *T. granulosum* culture filtrate. Samples (5 ml) of the culture filtrate were adjusted to the desired pH by titration with either 0.1 M HCl or 0.1 M NaOH on a Sargent pH-stat. After incubating at 37° for 30 min, an aliquot equivalent to 3 ml of culture filtrate was withdrawn and added to 5 ml of 0.2 M phosphate buffer (pH 7.8). The caseinolytic activity was measured by the standard assay procedure (see MATERIALS AND METHODS).

Fig. 2. The effect of pH on the proteolytic activity of *T. granulosum* culture filtrate and purified enzyme. For assay conditions see MATERIALS AND METHODS. The buffers used were: 0.05 M HCl–NaCl (pH 2.5–3.0), 0.05 M sodium acetate–acetic acid (pH 3.5–5.5), 0.05 M Sörensen's phosphate (pH 6.0–8.0), 0.05 M sodium borate–boric acid (pH 8.5–9.8), and 0.05 M sodium carbonate–sodium carbonate–sodium bicarbonate (pH 10.0–11.0). ●—●, 0.3 ml of culture filtrate with the hemoglobin substrate; ○- -○, 0.3 ml of culture filtrate with a casein substrate, △- -△, approx. 0.5 μg of protease with a casein substrate.

Optimum ionic strength

The optimum ionic strength for proteolysis was determined by measuring the caseinolytic activity of the enzyme in phosphate buffer containing varying concentrations of NaCl. A series of solutions of increasing ionic strength was prepared by the addition of calculated amounts of NaCl to 0.007 M Sörensen's phosphate buffer (pH 7.8), 1 mM $MgCl_2$. The protease exhibited maximal caseinolytic activity at $I = 0.10$.

Effect of temperature

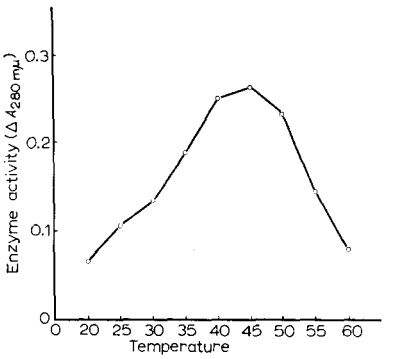
Fig. 3 depicts the influence of temperature on the caseinolytic activity of the protease. The rate of hydrolysis reached a maximum at 45°.

Effect of metal ions

The enzyme was activated by Ba^{2+}, Mg^{2+} and Ca^{2+}, and by Zn^{2+} in low concentration. EDTA deactivated the protease and various other metal salts were inhibitory (Table II).

Amino acid composition

The results of a single amino acid analysis of the acid hydrolysate of the protease is presented in Table III. Tryptophan was completely destroyed under the conditions of the hydrolysis. Fig. 4 shows the ultraviolet absorption spectra of the protease in 0.1 M sodium acetate buffer (pH 6.0) and in 0.1 M NaOH (pH 13.0). The enzyme, in acetate buffer, showed one absorption maximum at 274 mμ. In alkali, the



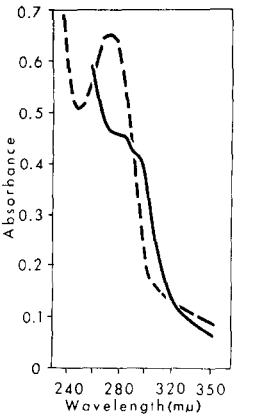


Fig. 3. The effect of temperature on the caseinolytic activity of *T. granulosum* protease. See MATERIALS AND METHODS for the assay procedure.

Fig. 4. The ultraviolet spectra of *T. granulosum* protease; — — —, 0.1 M sodium acetate–acetic acid buffer (pH 6.0); ———, in 0.1 M NaOH (pH 13.0).

TABLE II

THE EFFECT OF METAL IONS ON CASEINOLYTIC ACTIVITY

See MATERIALS AND METHODS for assay conditions. The metal ions were added as chloride salts to aliquots of the protease in 0.05 M veronal buffer (pH 7.8) and kept at 4° for 16 h before assayed. Samples were then warmed to 37° for 5 min before assayed.

Ion	*Activity (%)*		
Final concn:	10^{-2} *M*	10^{-3} *M*	10^{-4} *M*
Control	100	100	100
Ca^{2+}	109	106	83
Ba^{2+}	145	137	129
Mg^{2+}	107	125	68
Mn^{2+}	0	51	68
Zn^{2+}	—	18	131
Ni^{2+}	—	12	7
Co^{2+}	—	50	64
EDTA	0	0	4

ionized tyrosine absorbs at 293 mμ. The slope of a line drawn tangent to the maxima at 274 mμ and 293 mμ furnished data for calculating the tryptophan to tyrosine molar ratio. The large amount of tryptophan indicated is in agreement with the high $E^{1\%}_{1cm}$ value for the enzyme.

Molecular weight

A graph was constructed of the elution rates of 5 reference proteins from a Sephadex G-100 column by plotting the elution volume to void volume (V_e/V_0) ratios against the logarithm of the molecular weights. In comparison, a V_e/V_0 ratio of 1.68 for the protease corresponded to a molecular weight of 34 300.

TABLE III

AMINO ACID COMPOSITION OF *T. granulosum* PROTEASE

Amino acid	*μmole/mg protein**	*mole/mole histidine*	*moles/mole protein***
Asp	0.46	7.7	46
Glu	0.16	2.7	16
Gly	0.34	5.7	34
Ala	0.27	4.5	27
Val	0.09	1.5	9
Leu	0.15	2.5	15
Ile	0.08	1.3	8
Ser	0.19	3.2	19
Thr	0.22	3.7	22
Cys***	undetected		
Met	trace		
Pro	0.17	2.8	17
Phe	0.13	2.2	13
Tyr	0.14	2.3	14
Trp		4.1§	25§
His	0.06	1.0	6
Lys	0.09	1.5	9
Arg	0.14	2.3	14
			294

* Analysis was on the lyophilized sample.

** Taking the molecular weight of protein to be 34 300. The sum of the acids and termina water is 33 266.

*** Undetected in the acid hydrolysate of 18 h at 100°. Analysis of the cysteic acid in a performic acid-oxidized sample was not performed.

§ From the absorption spectrum of the enzyme in 0.1 M NaOH.

Substrate specificity

The protease hydrolyzed the A and B chains of oxidized insulin. On two-dimensional thin-layer chromatograms (solvents: *n*-butanol–acetic acid–water (80:20:20, by vol.); phenol–water (83:17, by vol.)) digestion solutions indicated 11 discernible spots from the A chain and 19 from the B chain. No spot could be verified as an amino acid by comparison with standard samples. The protease did not hydrolyze L-tyrosylglycine, L-phenylalanylglycine, glycyl-L-phenylalanine, L-serylglycine, DL-alanyl-DL-serine, L-valylglycine, L-leucyl-L-tyrosine and oxidized glutathione.

DISCUSSION

It was necessary to grow *T. granulosum* on a keratin substrate in order to induce the production of the extracellular protease responsible for the solubilization of hair. Earlier attempts to cultivate the fungus on a large scale on a 1% neopeptone–yeast medium resulted in good growth. However, fractionation of the culture liquor furnished only a small yield of the alkaline protease. Presumably, the rich medium suppressed the generation of the extracellular protease.

The purification of the enzyme was attempted with large batches of enzyme concentrate on three different occasions. Each time, variations in the order of procedures were carried out in order to reduce the number of operations since some steps

were invariably accompanied by severe losses of activity. The greatest losses of activity occurred toward the end of the purification procedures indicating that the stability of the enzyme decreased with purity.

The *T. granulosum* protease possesses distinctive characteristics although its molecular weight of 34 300 is similar to values reported for other proteases isolated from filamentous fungi[13–16]. The protease is characterized by its high tryptophan content. The ratio of tryptophan to tyrosine, almost 2:1, is an outstanding aspect of its amino acid composition compared with analyses of proteolytic enzymes of filamentous fungi and Streptomyces species [13,16,17].

Both culture filtrate and purified enzyme had the same optimum pH of 9.5–9.8 for caseinolytic activity. This optimum lies between those observed by BERGKVIST[18] for the protease of *Aspergillus oryzae* and by SINGH AND MARTIN[14] for the protease of *Penicillium cyano-fulvum*. The temperature optimum of 45° at pH 7.8 is close to that of the protease of *P. chrysogenum*[19] and identical with those of both proteases of *Glicladium roseum*[20].

The caseinolytic activity of the protease was increased by calcium and magnesium salts and particularly by barium ions. Zinc, in low concentrations, activated the enzyme, whereas cobalt, manganese and nickel inhibited. In contrast, both proteases of *G. roseum*[20], the protease of *P. cyano-fulvum*[14] and the protease I of *A. oryzae*[18], all alkaline proteases of molds, showed negligible or no inhibition in the presence of EDTA. Furthermore, the protease of *P. cyano-fulvum* was strongly inhibited by barium ions.

The aforementioned data denote that the protease of *T. granulosum* is not identical to any of the alkaline proteases of the other molds.

The protease did not hydrolyze a series of peptides as reported for the keratinase of *Streptomyces fradiae*[21]. It did degrade the A and B chains of oxidized insulin; however, no amino acids were found among the products of hydrolysis.

Our findings indicate that *T. granulosum*, in its parasitic growth phase, uses the protease as its first biochemical tool to utilize the hair as growth medium. Since no amino acids were detected in the reaction mixture of hair incubated with the protease, other enzymes are probably responsible for further hydrolysis.

ACKNOWLEDGEMENTS

We wish to thank Dr. M. P. BRIGHAM and Mr. EARL PEARSON, Section of Surgical Research, Department of Surgery, for the amino acid analysis.

This investigation was supported by the John A. Hartford Foundation, Inc., New York, and in part by Grant 5 RO1 GM 10212 from the U.S. Public Health Service. W.C.D. was a U.S. Public Health Research Fellow 1 F3 AM-31, 782-01.

REFERENCES

1 A. MACFADYEN, *J. Pathol. Bacteriol.*, 3 (1896) 176.
2 L. ROBERTS, *J. Pathol. Bacteriol.*, 3 (1896) 300.
3 L. ROBERTS, *Brit. Med. J.*, 1 (1899) 13.
4 E. BODIN, *Ann. Dermatol. Syphilig.*, 8 (1907) 585.
5 C. N. D. CRUICKSHANK AND M. D. TROTTER, *Nature*, 177 (1956) 1085.
6 F. W. CHATTAWAY, D. A. ELLIS AND A. J. E. BARLOW, *J. Invest. Dermatol.*, 41 (1963) 31.
7 M. J. KUNITZ, *J. Gen. Physiol.*, 30 (1947) 291.

8 M. L. ANSON, *J. Gen. Physiol.*, 22 (1939) 79.
9 F. G. BLIGH AND W. J. DYER, *Can. J. Biochem. Physiol.*, 37 (1959) 11.
10 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
11 P. ANDREWS, *Biochemistry*, 96 (1965) 595.
12 W. L. BENCZE AND K. SCHMID, *Anal. Chem.*, 29 (1957) 1193.
13 J. SAWADA, *Agr. Biol. Chem. Tokyo*, 30 (1966) 393.
14 K. SINGH AND S. M. MARTIN, *Can. J. Biochem. Physiol.*, 38 (1960) 969.
15 A. A. BURGUM AND J. M. PRESCOTT, *Arch. Biochem. Biophys.*, 111 (1965) 391.
16 T. HOFMAN AND R. SHAW, *Biochim. Biophys. Acta*, 92 (1964) 543.
17 A. R. SUBRAMANIAN AND G. KALNITSKY, *Biochemistry*, 3 (1964) 1868.
18 R. BERGKVIST, *Acta Chem. Scand.*, 17 (1963) 154.
19 S. YOSHIMURA, *Mem. Hyogo Univ. Agr.*, 13 (1962) 1.
20 T. KISHIDA AND S. YOSHIMURA, *Agr. Biol. Chem. Tokyo*, 30 (1966) 1183.
21 W. J. NICKERSON AND S. C. DURAND, *Biochim. Biophys. Acta*, 77 (1963) 87.