BBA 65595

# MULTIPLE PROTEOLYTIC ENZYMES OF *STREPTOMYCES FRADIAE* PRODUCTION, ISOLATION, AND PRELIMINARY CHARACTERIZATION

KAZUYUKI MORIHARA, TATSUSHI OKA AND HIROSHIGE TSUZUKI
*Shionogi Research Laboratory, Shionogi and Co. Ltd., Fukushima-ku, Osaka (Japan)*

(Received November 4th, 1966)

## SUMMARY

*Streptomyces fradiae* exhibited the highest potency in the production of proteolytic enzymes among about 500 strains tested, all of Streptomyces genus. It was found that at least five proteinases (peptide peptidohydrolases) and two peptidases such as leucine aminopeptidase and carboxypeptidase were produced by the organism, which were fractionable by column chromatography on carboxymethyl-cellulose. Various physicochemical and enzymic properties were studied. One of the enzymes that was easily crystallized showed similar characters to those of "keratinase", previously discovered by NICKERSON, NOVAL AND ROBISON, and produced by the same organism in a keratin–salt medium. A further comparative study on the proteolytic enzyme system of *S. fradiae* cultured in keratin–salt medium and that cultured in the ordinary non-keratinic medium disclosed no difference between them.

## INTRODUCTION

While investigating about 500 strains of Streptomyces genus in order to obtain a strain possessing an ability to produce a high potency of proteolytic enzymes, we found the highest production by using *Streptomyces fradiae* and an ordinary nutrient medium in a shaking culture. It was already known[1,2] that the organism produced a keratinolytic enzyme termed "keratinase" in a keratin–salt medium. The enzyme had been regarded as a proteolytic enzyme[3], because the crystalline enzyme of homogeneous nature showed a high proteolytic activity against hemoglobin. In order to clarify the type of proteolytic enzyme produced by *S. fradiae* in the ordinary nutrient medium, the following study was undertaken, the result of which disclosed at least five proteinases (peptide peptidohydrolases) and two peptidases produced in the medium, one of which was seemed to be identical with "keratinase" produced by the same organism in a keratin–salt medium.

## MATERIALS AND METHODS

### *Organisms and culture*

About 150 type cultures of various species and 350 cultures of unidentified ones belonging to the Streptomyces genus and isolated from soil were kindly donated by Drs. NISHIMURA AND MAYAMA of this laboratory. Except as specified, the following cultural method was adopted. Basal medium was composed as follows: 3% potato starch, 1% soybean meal, 0.5% corn steep liquor, 0.5% glycerol, 0.3% NaCl, 0.35% $CaCO_3$, and 0.1% yeast extract, pH 7.0. Strains were cultured in 100 ml of the medium in 500-ml Sakaguchi flasks, which were shaken on a reciprocal shaker (130 rev./min, 7 cm amplitude) for 3 to 5 days at 28°.

### *Enzymes, substrates, inhibitors, and others*

Keratinase concentrate "M-zyme" (Lot. No. 63 494, 30 000 keratinase units/ml) was kindly donated by Merck and Co. Crystalline trypsin (EC 3.4.4.4) was purchased from Mochida Pharmaceutical Co., Tokyo, crystalline $\alpha$-chymotrypsin (EC 3.4.4.5) from Sigma Chemical Co., St. Louis, and crystalline proteinase of *Bacillus subtilis* (Nagarse) from Nagase Industrial Co., Osaka.

Casein (Hammarsten) was purchased from Wako Pure Chemical Industries Co., Osaka. Elastin (C grade, sieved 50 mesh) was obtained from Calbiochem. Various synthetic peptides* were supplied by the Peptide Center at the Institute for Protein Research of Osaka University. Z–Gly–Leu–$NH_2$ and Phe–$NH_2$ were prepared in this laboratory. Poly-amino acids were purchased from Mann Research, New York. Wool, clipped from ewes at the Osaka Prefectural Livestock Experimental Station, was washed with water and chloroform, then dried in the air. As substrate, the wool was cut with scissors into pieces about 2–3 mm long.

Crystalline soybean trypsin inhibitor (recrystallized, 5 times) was obtained from Mann Research, New York. Purified potato inhibitor was prepared by the method of MATSUSHIMA[4]. Diisopropylphosphofluoridate (DFP) was obtained from Sigma Chemical Co., St. Louis. DEAE-cellulose (Serva, 0.81 mequiv/g), CM-cellulose (Serva, 0.75 mequiv./g), and Sephadex G-100 (Pharmacia) were purchased from Seikagaku Kenkyusho, Tokyo. Crystalline ovalbumin was kindly donated by Dr. H. INOUE of this laboratory. Cytochrome *c* from horse heart (Type II, Sigma Chemical Co.) and *p*-chloromercuribenzoic acid (PCMB) were obtained from Nakarai Chemicals Ltd., Kyoto.

### *Estimation of enzymic activity*

Proteolytic activity, except when specified, was determined by digestion of 1 ml of 2% casein solution (pH 7.4) with 1 ml of enzyme solution (suitably diluted with 0.1 M phosphate of pH 7.4) at 40° for 10 min and estimating the liberated tyrosine by Folin–Ciocalteu reagent, as described previously[5]. The absorbance at 670 m$\mu$ was read. The specific proteolytic activity (P.U.) is expressed as mg of tyrosine released per min per mg of enzyme (or ml of enzyme solution). Protein

* Abbreviated designations of amino acid derivatives and peptides are used according to tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature. All the amino acids were L-form, except glycine.

content of enzyme solutions was determined by the method of LOWRY *et al.*[6] using crystalline ovalbumin as standard.

Elastolytic activity was determined as follows. The reaction mixture, containing 20 mg of elastin, 1 ml of 0.1 M Tris buffer (pH 8.5), 1 ml of enzyme solution (purified enzyme), and 1 ml of distilled water, was shaken at 40° for 1 h. To the reaction mixture, 2 ml of 0.7 M phosphate buffer (pH 6) was added, and the mixture was then filtered. Protein content of the filtrate was measured by the colorimetric method of LOWRY *et al.*[6], whereby the elastin dissolved was determined. The unit for elastolytic activity (E.U.) was expressed as mg of elastin dissolved per h per mg of enzyme.

Keratinase activity was assayed by a wool-digestion method of NICKERSON, NOVAL AND ROBISON[2], whereby the amount of protein solubilized during the incubation period was estimated spectrophotometrically at 280 mμ.

Activities on various synthetic substrates were determined as follows. Esterase activity was examined by titration with 0.01 M NaOH in the presence of formaldehyde (formol titration)[7] or ethanol (alcohol titration)[8,9]. Either the $NH_3$ or α-amino acid released was determined using CONWAY's apparatus[10] or by the ninhydrin method[11].

## RESULTS

### *Production of proteinase by S. fradiae*

Among about 500 strains of various species of Streptomyces tested, *S. fradiae* ATCC 3535 and U-260 were found to have a much higher production potency either in proteolytic or elastolytic activity than the others, although a considerable production potency of those enzymes was also observed in *Streptomyces rimosus, Streptomyces flavovirens, etc.* Effects of various cultural media on the proteinase production of *S. fradiae* were determined. It was found that high concentrations of starch (over 1%) were required to promote the production, whereas those of glucose inhibited it. The addition of soybean meal above 1% did not usually increase the production but rather inhibited it. Therefore the basal medium described under METHODS was used. The effect of aeration was also determined. The highest activity was achieved in a 2-l erlenmeyer flask containing 300 ml of the medium which was shaken on a rotatory shaker (188 rev./min, 6.5 cm amplitude) for 3 days at 28°. The proteolytic activity thus produced in the fermentation broth was $5 \cdot 10^{-3}$–$10 \cdot 10^{-3}$ P.U./ml.

### *Crystallization of proteinase from S. fradiae*

The fermentation broth of *S. fradiae* ATCC 3535 which reached the highest proteolytic activity as aforementioned was centrifuged and subjected to such treatment as salting out with $(NH_4)_2SO_4$ (0.6 saturation) and fractional precipitation with acetone (0.5–2 vol. precipitation fraction) at about 4° to obtain a crude proteinase solution. The dark brown enzyme solution thus obtained was treated with DEAE-cellulose, which was originated by NICKERSON AND DURAND[3] for crystallization of keratinase of *S. fradiae*. A suitable amount of DEAE-cellulose was mixed with the above crude enzyme solution which had previously been dialyzed against 0.01 M Tris buffer (pH 7). The mixture was agitated and then filtered. The dark-brown cellulose residue was washed twice with 0.01 M Tris buffer (pH 7). The washed

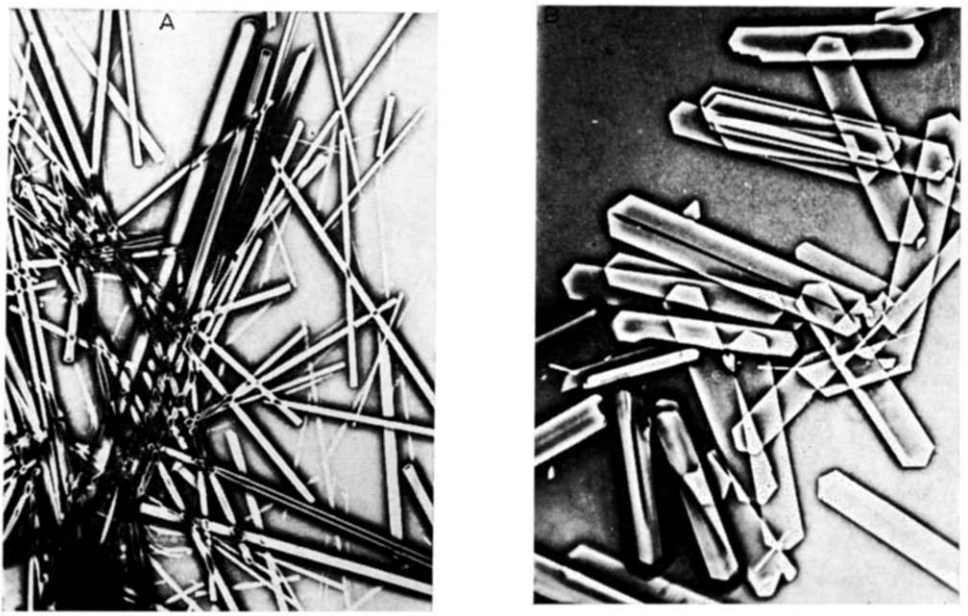


Fig. 1. Crystalline proteinase of *S. fradiae* crystallized slowly (A) (× 375) or rapidly (B) (× 375).

residue contained negligibly small proteolytic activity. The colorless filtrate thus obtained was concentrated *in vacuo* in a rotatory evaporator below 35°, and then dialyzed against 0.05 M Tris buffer (pH 9) for 16 h in a cold place. After the residue had been discarded by centrifugation, the clear supernatant was further dialyzed against running distilled water for two days. After centrifugation, the clear supernatant was allowed to stand at room temperature. Within a few hours, beautiful crystals were formed (Fig. 1A). Rapidly crystallized enzyme always took the form of rods (Fig. 1B). The proteolytic activity was determined after dissolution of the crystals; the solution was then subjected to the alkaline treatment described below. The yield of the crystalline enzyme was only 5.6% from the decolorized material treated with DEAE-cellulose.

*Column chromatography of the proteolytic enzymes from S. fradiae on CM-cellulose*

The low yield of crystalline proteinase as mentioned above suggested the existence of another type of proteinase together with the crystalline enzyme. This was confirmed in the following manner. The crude enzyme preparation of *S. fradiae* ATCC 3535 was subjected to column chromatography on CM-cellulose. Fig. 2 shows that four proteinase fractions (Fractions I–IV) were separated by chromatography, of which Fraction III was found to have a high elastolytic activity. Peptidase activity on either Leu–Gly or Z–Gly–Leu was detected in the proteinase part of Fraction I. Fraction I was further fractionated into two components of proteinases (Fractions Ia and Ib) and one peptidase fraction (fraction tubes 35–60) by column chromatography of B of Fig. 2. The recovery of proteolytic activity was 71% from A, and 81% from B. A crystalline proteinase obtained as above was applied to the column of A in Fig. 2, and was found to correspond to Fraction II. Five proteinase

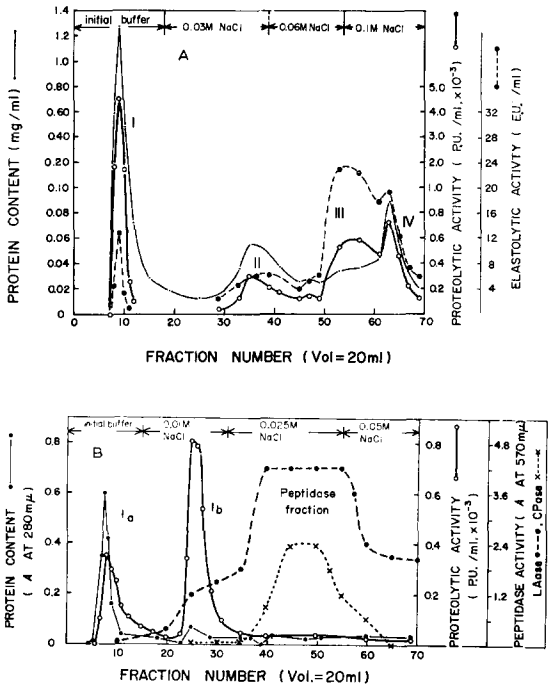


Fig. 2. Column chromatography of the proteolytic enzymes from *S. fradiae* on CM-cellulose. A column of CM-cellulose (3 cm × 21 cm) was used, and the flow rate was adjusted to about 0.5 ml/cm² per min. The volume of each fraction collected was 20 ml. Elution was carried out by a stepwise increased concentration of NaCl, as seen in the figure. The protein content of each fraction tube was determined by the method of LOWRY *et al.*[6] or by the absorbance at 280 mμ. The determination of either proteolytic or elastolytic activity is described in the text. Peptidase activities were determined as follows. A reaction mixture containing 0.1 ml of 0.01 M substrate (Leu–Gly or Z–Gly–Leu), 0.4 ml of 0.1 M Tris buffer (pH 8), 0.1 ml of 0.1 M cation ($CaCl_2$ in the former substrate, and $CoCl_2$ in the latter), and 0.4 ml of each fraction tube was kept for 15 min at 40°. Immediately, to the reaction mixture containing the former substrate there was added 0.5 ml of 0.2 M citrate buffer (pH 5) containing 0.01 M EDTA, whereas that containing the latter substrate was kept in a boiling-water bath for 10 min to stop the reaction. The ninhydrin value was determined as usual, and each activity was shown by the absorbance at 570 mμ. A, The column was buffered with 0.05 M phosphate at pH 6, and 200 mg of lyophilized crude enzyme preparation of *S. fradiae* ATCC 3535 (salted out with $(NH_4)_2SO_4$, precipitated by acetone, and dialyzed against the same buffer) was applied. B, The column was buffered with 0.001 M Tris–maleate buffer (pH 6) containing 0.002 M calcium acetate. The fraction I of A of this figure was concentrated and dialyzed against the same buffer, and the dialyzate (46.7 mg as protein) was applied to the column. Abbreviations: LAase, leucine aminopeptidase; CPase, carboxypeptidase.

fractions and one peptidase fraction thus obtained were concentrated *in vacuo* below 35° using a rotary evaporator. The concentrate was dialyzed against distilled water or suitable buffer solution; the dialyzate was centrifuged, and the clear supernatant was used for the following examination.

*Physicochemical properties of the various proteinases from S. fradiae*

*Solubility.* The solubility of each fraction (II, III and IV) in neutral solution was low. When a concentrated solution of 0.5% or higher was dialyzed against such buffer solution as used for the following experiment, an amorphous precipitate was occasionally formed. The following physico-chemical properties were therefore

determined on an enzyme solution of comparatively low concentration after the removal of amorphous residues by centrifuging. The crystalline materials were sparingly soluble in neutral solution. Dissolution was possible either by raising the pH to 11.5, or by decreasing it below 4. For the following experiment, the crystals were suspended in 0.1 M Tris buffer (pH 9) containing 0.001 M $CaCl_2$. The suspension was cooled to about 4° and the pH adjusted to 11.5 with 0.1 M NaOH, which was immediately lowered to a suitable pH range or the suspension was dialyzed against a buffer solution. Ca ions exhibited a stabilizing effect for all the enzyme preparations. Fractions Ia and Ib were easily soluble in neutral solution.

*Sedimentation and diffusion studies.* The sedimentation study was made with a Hitachi UCA-I analytical ultracentrifuge. A buffer solution that was used contained 0.01 M Tris (pH 7), 0.001 M $CaCl_2$ and 0.175 M NaCl. The enzyme concentration and the rotor speed were 0.34–0.65% and about 60 000 rev./min, respectively. The run was carried out for 90 min at 20°. The results indicated that each proteinase (Fractions Ib, II, IV, and crystalline proteinase) was homogeneous in nature with a single sedimenting boundary. From the pattern, the $s_{20,w}$ of Fraction Ib was calculated as 2.88 S, that of both Fraction II and the crystalline proteinase was 2.24 S, and that of Fraction IV was 2.54 S. The determination of Fraction III was impossible, because the peak diminished during the run, possibly owing to the low concentration.

The diffusion measurements of crystalline proteinase were performed with a Neurath type cell at 27° in 0.01 M Tris buffer (pH 7) containing 0.001 M $CaCl_2$ and 0.175 M NaCl. The maximum height–area method was employed to calculate the diffusion constant, which was then corrected to the value in water at 20°. The diffusion constant ($D_{20,w}$) was found to be $11.49 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$. Assuming that the partial specific volume is 0.72 $cm^3 \cdot g^{-1}$, the molecular weight of the crystalline material was calculated to be 17 700.

*Gel-filtration study.* To determine the molecular weight of Fraction III, the gel-filtration method[12] was adopted using Sephadex G-100. The fraction was contaminated with a small quantity of Fraction IV, of which the activity was specifically

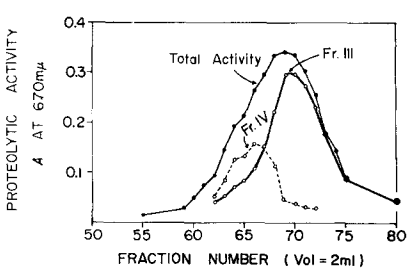


Fig. 3. Gel filtration of Fraction III proteinase from *S. fradiae* using with Sephadex G-100. The column (1.75 cm × 66 cm) of Sephadex G-100 had previously been equilibrated with the buffer solution containing 0.05 M Tris buffer (pH 7.5), 0.005 M $CaCl_2$, and 0.1 M KCl. The Fraction III was dialyzed against the same buffer, and 0.5 ml of the dialyzate ($5.42 \cdot 10^{-3}$ P.U.) was applied to the column. Elution was carried out by the same buffer solution, and the flow rate was adjusted at 3.3 ml/$cm^2$ per h. The volume of each fraction tube collected was 2 ml. The proteolytic activity of each fraction tube was determined before and after treatment with soybean trypsin inhibitor (0.05 mg inhibitor per ml enzyme solution). The treatment with the inhibitor was carried out at 40° for 30 min. The proteolytic activity not affected by soybean trypsin inhibitor was regarded as that of Fraction III, whereas that affected by the inhibitor was Fraction IV.

inhibited by soybean trypsin inhibitor as will be described later. The pattern of Fraction III was regarded as having a proteolytic activity unaffected by soybean trypsin inhibitor (Fig. 3). The proteolytic activity inhibited by soybean trypsin inhibitor, corresponding to Fraction IV, is also shown in Fig. 3. From the comparative study using various pure proteins such as cytochrome *c*, soybean trypsin inhibitor, ovalbumin and polylysine, whose molecular weights were already known, the molecular weights of Fractions III and IV were determined as 14 000 and 16 500, respectively.

*Electrophoretic study.* The study was made in a 2-ml cell using the Hitachi Tiselius electrophoresis apparatus, Type HT-B. The electrophoresis buffer used was 0.01 M Tris buffer (pH 7 or 9), containing 0.001 M $CaCl_2$ and 0.175 M NaCl. The run was carried out for 120 min at 10 mA and 72 V at pH 7, or at 5 mA and 35 V at pH 9, and the temperature was 4°. Enzyme preparations such as Fractions II–IV and the crystals showed a homogeneous nature and moved to the cathode, maximally at pH 7, but slightly at pH 9. Fraction IV showed a considerable loss of proteolytic activity after electrophoresis at pH 9 even in the presence of $Ca^{2+}$. The enzyme preparation of Fraction Ib was also examined after treatment with DEAE-cellulose according to a method similar to that described in the section on purification and crystallization of proteinase, by which a small amount of acidic component adhering to the enzyme protein was removed. The preparation showed a homogeneous nature and an electrophoretic pattern similar to those of the other enzyme fractions mentioned above. These criteria indicated that their isoelectric points were around pH 9.

*Enzymic properties of various proteinase fractions from S. fradiae*

*Optimal pH.* Fig. 4 shows the optimal pH of various different proteinase fractions, that of Fraction Ia with casein as substrate was at pH 9–10, Fraction Ib at pH 11–11.5, Fraction II at 8.5–10, Fractions III and IV at 6.5–9, while that of Fraction III with elastin as substrate was at pH 9. Crystalline proteinase also showed the highest activity towards casein at pH 8.5–10, as similarly observed for Fraction II.

*Actions on various proteins or synthetic peptides.* Table I shows the enzymic activities of various proteinase fractions on different substrates. Fraction III possessed the highest activity towards either casein or elastin, which further showed the

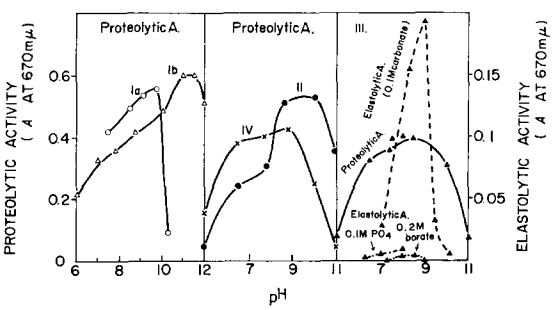


Fig. 4. Optimum pH of various proteinase fractions from *S. fradiae* against casein and elastin. Both casein solution (2%) and enzyme solution (suitably diluted) were adjusted to the respective pH value shown in the figure, and the reaction was begun by mixing both the solutions. Determination of elastolytic activity was carried out in the buffer solution of various pH values as shown.

TABLE I

ACTIVITY OF EACH PROTEINASE FRACTION FROM *S. fradiae* ON VARIOUS SUBSTRATES

Determination of either proteolytic or elastolytic activity is described in METHODS. Keratinolytic activity was determined by keeping a reaction mixture (10 ml) containing 70 mg of wool, 0.05 M Tris buffer (pH 9) and an enzyme preparation showing proteolytic activity of $2.5 \cdot 10^{-3}$ P.U. at 40° for 3 h. After filtration, the absorbance of the filtrate was read at 280 mμ. Activities against synthetic substrates were examined as follows. The reaction mixture of 1 ml containing substrate (either 0.016 M Bzl–Arg–$NH_2$, 0.01 M Bzl–Arg–OEt, or 0.02 M Ac–Tyr–OEt), a suitable amount of enzyme, and 0.05 M Tris buffer (pH 8) or 0.02 M Tris–acetate buffer (pH 7) (for the latter two substrates) was kept at 40° for 15 min. With Ac–Tyr–OEt, the reaction mixture contained 6% ethanol for the low solubility. The initial velocity (μmoles split/min per mg enzyme) was calculated.

| | *Proteinase fraction* | | | | | |
|---|---|---|---|---|---|---|
| | *Ia* | *Ib* | *II* | *III* | *IV* | *Crystals* |
| Proteolytic activity (P.U. × $10^{-3}$) | 4 | 4.5 | 5.3 | 16.0 | 8.2 | 4.7 |
| Elastolytic activity (E.U.) | — | 28 | 107 | 700 | 218 | 84 |
| Keratinolytic activity ($A$ at 280 mμ) | 0 | 0.039 | 0.209 | 0.205 | 0.118 | 0.212 |
| Activity against synthetic substrates (initial velocity, μmoles/min per mg enzyme) | | | | | | |
| Bzl–Arg–$NH_2$ | | 0* | 0 | 0 | 1.6 | 0 |
| Bzl–Arg–OEt | | 0.7* | 2.2 | 10.9 | 12.5 | 2.8 |
| Ac–Tyr–OEt | | 1.4* | 3.0 | 4.9 | 0.2 | 3.7 |

* Fraction I.

highest keratinolytic activity together with Fraction II or crystals. On the other hand, Fraction IV showed the highest activity towards both Bzl–Arg–$NH_2$ and Bzl–Arg–OEt which are known to be suitable substrates for trypsin. Crystalline trypsin had about half the specific activity of Fraction IV. The highest activity towards Ac–Tyr–OEt, a suitable substrate for chymotrypsin, was observed in Fractions II, III and crystals, whereas negligible activity was observed in Fraction IV. The specific activity of Fraction III was almost equal to that of crystalline chymo-

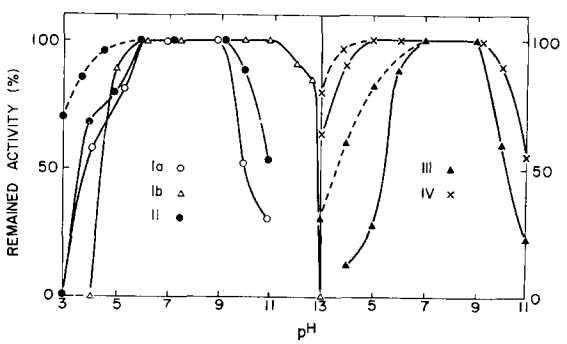


Fig. 5. pH stability of various proteinase fractions from *S. fradiae*. Various pH values at pH 3–6 were prepared by McIlvaine's citrate buffer, those at pH 7–8 by Sörensen's phosphate buffer, and those at pH 8–12.6 by Sörensen's glycinate buffer. As a solution of pH 13, 0.1 M NaOH was used. Each buffer solution containing each proteinase fraction (0.1 mg/ml) was kept in the presence (- - - -) or absence (————) of 0.01 M $CaCl_2$ for one night at room temperature. After reversing the pH value at pH 7.4, the residual proteolytic activity was determined.

TABLE II

THERMOSTABILITY OF VARIOUS PROTEINASE FRACTIONS FROM *S. fradiae*

Each proteinase fraction (0.1 mg/ml) containing 0.1 M Tris buffer (pH 7) was kept at various temperatures in the presence (+) or absence (−) of 0.01 M $CaCl_2$. After the treatment, the reaction mixture was immediately cooled, and the remaining proteolytic activity was determined.

| *Treatment* | $Ca^{2+}$ ($10^{-2}$ *M*) | *Fraction* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *I* | *Ib* | *II* | *III* | *IV* | *Crystals* |
| | | *(residual proteolytic activity, %)* | | | | | |
| 60°, 10 min | − | 98 | 84 | 58 | 9 | 9 | 60 |
| | + | 100 | 100 | 92 | 83 | 26 | 85 |
| 70°, 10 min | − | 66 | 63 | 2 | 0 | 0 | 0 |
| | + | 100 | 97 | 71 | 49 | 3 | 75 |
| 80°, 10 min | − | 34 | 49 | 0 | 0 | 0 | 0 |
| | + | 49 | 90 | 0 | 0 | 0 | 0 |
| 80°, 1 h | − | 7 | 0 | 0 | 0 | 0 | 0 |
| | + | 42 | 72 | 0 | 0 | 0 | 0 |

trypsin. None of the fractions showed any activity towards Z–Glu–Tyr. Among various poly-amino acids such as poly-L-aspartic acid, poly-L-glutamic acid, poly-L-tyrosine, poly-L-lysine, poly-L-proline, and polysarcosine, only poly-L-lysine was hydrolysed. Paper chromatography of the digest indicated that the main products of catalysis by either Fractions I, II or crystals were lysine dimer and trimer, whereas those produced by Fractions III and IV were the monomer and dimer.

*pH- and thermostability.* A study was made on the stability of proteinase fractions at various pH ranges (Fig. 5). The stability of Fraction Ib in the alkaline pH range was truly remarkable: the enzyme was almost stable even at pH 12. The other fractions such as Fractions II, III and especially IV had considerable stability at acidic pH ranges in the presence of $Ca^{2+}$. The thermostability of each proteinase fraction was also studied. Table II shows that Fraction Ib is a thermostable enzyme. After treatment at 80° for 1 h in the presence of $Ca^{2+}$ 72% of the activity remained. Among various metal ions examined, $Ca^{2+}$ and $Sr^{2+}$ showed the highest stabilizing effects on the heat treatment of the enzyme. The other proteinase fractions were found to be thermo-unstable, although $Ca^{2+}$ showed a considerable stabilizing effect against heat-treatment.

*Effect of various inhibitors.* Inhibition effects of various inhibitors on each proteinase fraction are summarized in Table III, which shows that all the proteinase fractions together with the crystalline material were inhibited almost completely by either DFP or potato inhibitor. Fractions III and IV were inhibited by EDTA to a considerable extent. Soybean trypsin inhibitor caused a considerable inhibition of Fraction IV, which was almost doubled after the rechromatography of the fraction. The inhibition by 0.1 M NaCl on elastolytic activity of Fraction III was insignificant. PCMB did not affect the enzymic activity of Fraction Ib.

*Enzymic properties of leucine aminopeptidase and carboxypeptidase from S. fradiae*

*Optimal pH.* Fig. 6 shows the optimal pH for leucine aminopeptidase and carboxypeptidase. The optimal pH for leucine aminopeptidase was 10, and for carboxypeptidase 7.

TABLE III

EFFECTS OF VARIOUS INHIBITORS FOR ENZYMIC ACTIVITY OF EACH PROTEINASE FRACTION FROM *S. fradiae*

Each proteinase fraction ($0.5 \cdot 10^{-3}$ P.U./ml) containing 0.1 M Tris buffer (pH 8) and various inhibitors as shown in the table was kept at 40° for 30 min. The residual proteolytic (P) or elastolytic (E) activity was determined as usual.

| *Inhibitor* | *Residual activity (%)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Ia* | *Ib* | *II* | *III* | | *IV* | *Crystals* |
| | *P* | *P* | *P* | *P* | *E* | *P* | *P* |
| EDTA ($10^{-3}$ M) | 94 | 93 | 87 | 38 | 31 | 47 | 95 |
| DFP ($5 \cdot 10^{-3}$ M) | 22 | 10 | 5 | 10 | 0 | 9 | 1 |
| Soybean trypsin inhibitor (enzyme:inhibitor: 1:3 by wt.) | 100 | 100 | 100 | 100 | — | 41 (20)* | 100 |
| Potato inhibitor (enzyme:inhibitor: 1:3 by wt.) | 28 | 9 | 21 | 12 | 20 | 13 | 5 |
| NaCl ($10^{-1}$ M) | — | — | — | — | 95 | — | — |
| PCMB ($10^{-3}$ M) | — | 83 | — | — | — | — | — |

* Rechromatographed preparation was used for the determination.

*pH- and thermostability.* The pH stabilities of both peptidases were determined in similar conditions (Fig. 5). Leucine aminopeptidase was somewhat stable at alkaline pH (pH 7–11), whereas carboxypeptidase was more stable at acidic pH (pH 4–8). The thermostabilities of both peptidases were determined. Leucine aminopeptidase was thermostable regardless of the presence or absence of $Ca^{2+}$: no decrease in enzymic activity was observed in the treatment of leucine aminopeptidase at 80° for 1 h. On the other hand, carboxypeptidase was completely inactivated by treatment at 70° for 10 min.

*Substrate specificity.* Substrate specificity of each peptidase was determined

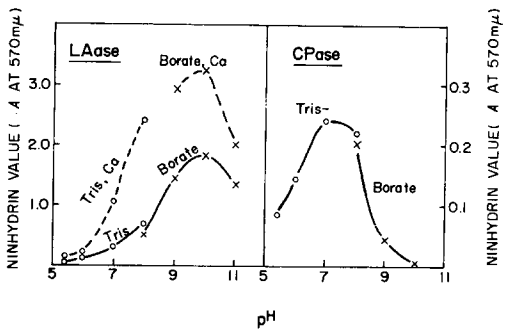


Fig. 6. Optimum pH of leucine aminopeptidase (LAase) and carboxypeptidase (CPase) from *S. fradiae*. The reaction mixture (1 ml), containing 0.01 M substrate (Leu–Gly for leucine aminopeptidase, and Z–Gly–Phe for carboxypeptidase), 0.05 M buffer solution of various pH values as shown in the figure, and a suitable amount of enzyme from peptidase fraction, was kept at 40° for 15 min. In the case of leucine aminopeptidase, the reaction was performed in the presence or absence of $Ca^{2+}$ ($10^{-2}$ M).

TABLE IV

SUBSTRATE SPECIFICITIES OF LEUCINE AMINOPEPTIDASE AND CARBOXYPEPTIDASE FROM *S. fradiae*

The reaction mixture of 1 ml containing 0.05 M Tris buffer (pH 8), a suitable amount of peptidase fraction, and 0.05 M (for leucine aminopeptidase) or 0.001 M (for carboxypeptidase) substrate was kept at 40° for 15 min. $CaCl_2$ (0.005 M) was added to the former reaction mixture, and $CoCl_2$ (0.01 M) to the latter. The activity is expressed by the initial velocity (μmoles split/min per mg enzyme). The arrows in the figure show the peptide bond hydrolyzed. The determination was carried out by paper chromatography of the digest and comparison with the authentical compounds.

| *Substrate* | *Leucine aminopeptidase (μmoles/min per mg enzyme)* | *Substrate* | *Carboxypeptidase (μmoles/min per mg enzyme)* |
|---|---|---|---|
| Leu–$NH_2$ | 98.7 | Z–Gly–Phe | 0.65 |
| Phe–$NH_2$ | 28.7 | Z–Gly–Phe–$NH_2$ | 0.52 |
| Leu–Gly | 406.0 | Z–Gly–Leu | 4.89 |
| Leu–Gly–Gly | 1165.0 | Z–Gly–Leu–$NH_2$ | 0.22 |
| Gly–Leu | 21.0 | Z–Glu–Phe | 0.29 |
| Gly–Phe–$NH_2$ | 54.2 | Z–Glu–Tyr | 0.59 |
| Gly–Gly | 10.8 | | |

(Table IV). The most suitable substrate for leucine aminopeptidase was Leu–Gly–Gly, and that for carboxypeptidase was Z–Gly–Leu.

*Effects of metal ions.* The effects of metal-chelating agents on both peptidase activities were studied. Leucine aminopeptidase was severely inhibited by chelating agents such as EDTA ($10^{-4}$ M), *o*-phenanthroline ($10^{-3}$ M), *etc.*, whereas a negligible

TABLE V

EFFECTS OF VARIOUS METAL IONS FOR PEPTIDASE ACTIVITIES OF LEUCINE AMINOPEPTIDASE AND CARBOXYPEPTIDASE FROM *S. fradiae*

An enzyme solution (0.5 ml) of 0.2 M Tris buffer (pH 8) containing a suitable amount of peptidase fraction was kept at 40° for 30 min in the presence or absence of a metal-chelating agent. Into the enzyme solution, 0.5 ml of various metal solutions ($2 \cdot 10^{-2}$ M) were added. Either leucine aminopeptidase or carboxypeptidase activity was determined by adding 0.1 ml of 0.1 M substrate (Leu–Gly or Z–Gly–Leu) to the reaction mixture and keeping at 40° for 15 min.

| *Metal* | *Leucine aminopeptidase ($A_{570\ m\mu}$)* | | *Carboxypeptidase ($A_{570\ m\mu}$)* | |
|---|---|---|---|---|
| | *Not treated* | *+EDTA ($10^{-4}$ M)* | *Not treated* | *+o-Phenanthroline ($5 \cdot 10^{-3}$ M)* |
| None | 1.48 | 0.12 | 0.300 | 0.031 |
| $ZnCl_2$ | 0.23 | 0.18 | 0 | 0.023 |
| $MgCl_2$ | 1.67 | 0.72 | 0 | 0.169 |
| $CaCl_2$ | 3.07 | 2.00 | 0.854 | 0.072 |
| $SrCl_2$ | 1.82 | 1.46 | 0.923 | 0.209 |
| $CoCl_2$ | 1.21 | 1.16 | 1.556 | 1.521 |
| $MnCl_2$ | 0.61 | 0.49 | 0.349 | 0.249 |

inhibition was observed on carboxypeptidase except with *o*-phenanthroline ($5 \cdot 10^{-3}$ M). A further study was made to determine whether metal ion is required for both peptidase activities using the enzyme preparation treated or not treated with some metal-chelating agent. Table V shows that leucine aminopeptidase requires either $Ca^{2+}$, $Sr^{2+}$ or $Co^{2+}$, whereas carboxypeptidase requires $Co^{2+}$. The optimal concentration of each metal ion was about $10^{-2}$–$10^{-3}$ M.

*Proteolytic enzyme system of keratinase concentrate, "M-zyme"*

*Keratinolytic activity of "M-zyme"*. The following experiment was carried out to facilitate a comparison between the above-mentioned protease preparation and a keratinase preparation which was manufactured in a keratin–salt medium by the same organism. A keratinase concentrate ("M-zyme"), supplied by Merck, was diluted with 20 volumes of 0.1 M phosphate buffer (pH 7.4) which corresponded to the original fermentation broth; this solution exhibited a proteolytic activity of $10 \cdot 10^{-3}$ P.U./ml. The activity was almost equal to that of the fermentation broth produced in the ordinary nutrient medium (non-keratinic) in our study. The keratinolytic activity was determined of both enzyme preparations which had previously been salted out with $(NH_4)_2SO_4$ (0.6 saturation) and dialyzed against distilled water. The result (Fig. 7) shows keratinolytic activities correlated with enzyme preparations

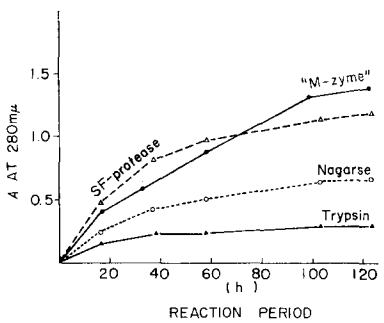


Fig. 7. Keratinolytic activity of "M-zyme" and other proteolytic enzymes. A reaction mixture of 10 ml containing 70 mg of wool, 0.05 M Tris buffer (pH 9), $5 \cdot 10^{-5}$ M $MgCl_2$, and each enzyme showing proteolytic activity of $5 \cdot 10^{-3}$ P.U., was kept at 37° for periods stated in the figure. For trypsin, a buffer at pH 8 was used. At the end of the reaction, the contents of each flask were filtered through a glass filter under gentle vacuum, and the increased absorbance at 280 mμ was determined. The remaining wool was further suspended in a new solution (10 ml) of each enzyme preparation as above. The increased absorbance at 280 mμ in exposure to five applications of each enzyme was determined. A comparative study was made using the crude enzyme preparation obtained from the fermentation broth of *S. fradiae* in an ordinary nutrient medium (abbreviated SF-protease) and others.

showing the same proteolytic activity. Evidently both enzymes, such as "M-zyme" and our protease preparation from the ordinary nutrient medium, possessed an almost equal activity towards native wool, the intensity of which was twice as high as that of "Nagarse" and about four times as high as that of trypsin.

*Crystallization of keratinase from "M-zyme", and its characteristic.* Crystalline keratinase was prepared from "M-zyme" according to the method described by NICKERSON AND DURAND[3]. The crystalline form was similar to that shown in Fig. 1B.

The solubility of the crystalline material was also low in neutral solution. The crystals were therefore dissolved by alkali treatment as described above. The sedimentation study was made by a similar method to that described above, and the $s_{20,w}$ was calculated to be 2.16 S. The value was a little lower than that (2.4 S) determined by NICKERSON AND DURAND[3]. The ultraviolet absorption spectrum of the crystalline keratinase (Fig. 8) coincided with that of the crystalline proteinase from *S. fradiae*. The $E^{1\%}_{280\,m\mu}$ of each preparation was 8.4. The value was also lower than that (10.42) determined by NICKERSON AND DURAND[3]. Electrophoretic study of the crystalline keratinase showed the isoelectric point to be about 9, identical with that of crystalline proteinase of *S. fradiae* as mentioned above. The enzymic characters of the keratinase were studied further including pH optimum, proteolytic activity towards various substrates, stability, behavior of various inhibitors, *etc.* No difference was observed in these characters between the crystalline keratinase and our crystalline proteinase.

*Column chromatography of "M-zyme" on CM-cellulose.* Further experiment was made to determine whether the multiple proteolytic enzymes produced in keratin–salt medium and in the ordinary non-keratinic medium are identical. "M-zyme" was dialyzed against distilled water, and treated with $(NH_4)_2SO_4$ (0.6 saturation) in the cold. The precipitate was removed by centrifugation, dialyzed against distilled water and lyophilized. The preparation showed a high absorbance in the ultraviolet spectral region for the complexing acidic component of the keratinase conjugate as had previously been described by NICKERSON *et al.*[2,3]. The ultraviolet absorption spectrum was compared with that of the enzyme preparation prepared from the ordinary nutrient medium (Fig. 8). The preparation, however, was not adsorbed on CM-cellulose unless the acidic component was removed by treatment with DEAE-

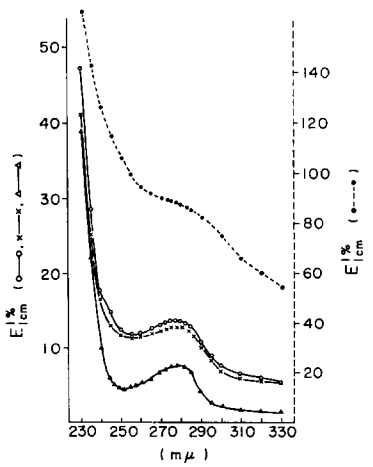


Fig. 8. Ultraviolet absorption spectrum of crystalline keratinase or partially purified preparations from "M-zyme". Determination was made on each enzyme preparation (0.4 mg protein per ml) containing 0.1 M Tris buffer (pH 8). "M-zyme" treated with $(NH_4)_2SO_4$ (0.6 saturation) (●— —●); "M-zyme" treated with DEAE-cellulose (○—○); and crystalline keratinase (△—△). For comparison, *S. fradiae* protease (prepared in ordinary nutrient medium) treated with $(NH_4)_2SO_4$ (0.6 saturation) (×—×) was determined.

cellulose. The ultraviolet absorbance of the treated solution is shown in Fig. 8. The decolorized solution was applied to a column of CM-cellulose previously buffered with 0.05 M phosphate at pH 6. The pattern of the chromatography was similar to that of Fig. 2A. Fraction I thus obtained was further separated chromatographically as for Fig. 2B. Five proteinase fractions (Fractions Ia, Ib, II–IV) and one peptidase fraction possessing leucine aminopeptidase and carboxypeptidase activities were obtained. The keratinolytic activity of each proteinase fraction was determined. The results established that both Fraction II (corresponding to crystalline keratinase) and III possessed a high keratinolytic activity similar to that of each corresponding enzyme obtained from ordinary nutrient medium (Table I). The study on other enzymic characters, showed that the five proteinases and two peptidases had properties identical with each corresponding enzyme from the ordinary nutrient medium.

## DISCUSSION

*S. fradiae* produced at least five proteinases (Fraction Ia, Ib, II–IV) and two peptidases (leucine aminopeptidase and carboxypeptidase) in the medium when the organism was cultured aerobically in a normal nutrient medium. The five proteinases were inhibited by either DFP or potato inhibitor and were active maximally in the alkaline pH range. These characters are similar to those of the alkaline proteinase of *Streptomyces griseus* described by HIRAMATSU AND OUCHI[13] and NARAHASHI AND YANAGITA[14]. Our enzymes, however, were intrinsically different from each other: Fraction Ib preserved its excellent stability not only at high temperature (80°) but also at alkaline pH range (pH 12). The enzyme in Fraction Ib had characteristics somewhat different from a thermostable proteinase obtained by MIZUSAWA, ICHISHIMA AND YOSHIDA[15], our proteinase not being affected by PCMB. Fraction II was easily crystallized, and Fraction III showed a remarkably high elastolytic activity, which activity was not affected by high concentrations of NaCl: hence it differed from the other known elastase preparations[16–18] except Flavobacterium elastase[19]. Fractions II and III possessed chymotrypsin-like characters showing high activity on Ac–Tyr–OEt. On the contrary, Fraction IV exhibited trypsin-like characters, showing high activity on either Bzl–Arg–$NH_2$ or Bzl–Arg–OEt and being inhibited by soybean trypsin inhibitor. The leucine aminopeptidase of the organism showed a high activity against Leu–Gly–Gly in comparison with Leu–Gly or Leu–$NH_2$ and was thermostable. The latter character resembles that of leucine aminopeptidase from *S. griseus*[14,20] but not that of the enzyme of *Streptomyces sioyaensis*[21]. The carboxypeptidase showed a manifold higher activity against Z–Gly–Leu than that of Z–Gly–Phe and was different from pancreatic carboxypeptidase A (EC 3.4.2.1)[22]. Both peptidases were regarded as metalloenzymes.

A further study on keratinase concentrate "M-zyme" disclosed that multiple proteolytic enzymes as produced in the ordinary nutrient medium were identical with those produced in the fermentation broth cultured on keratin–salt medium. Nevertheless, some differences were necessary in the purification procedures. With the "M-zyme", column chromatography on CM-cellulose was unsuccessful unless pretreatment by DEAE-cellulose was made, in order that an acidic component, that had been adherent to the keratinase preparation, was removed. On the other hand, such a pretreatment was not always required for the column chromatography of the

enzymes produced in an ordinary nutrient medium, which indicates that these enzymes were free from the acidic component. Treatment of "M-zyme" with DEAE-cellulose yielded a crystalline enzyme, which had hitherto been designated as keratinase by NICKERSON *et al.*[2,3], and was very similar to a crystalline enzyme obtained from the non-keratinic medium in this study. Both crystalline preparations corresponded to a proteinase fraction of Fraction II in column chromatography on CM-cellulose.

These results lead to the belief that the culture employing ordinary nutrient medium is more economical than that using a keratin medium to obtain multiple proteolytic enzymes including keratinase or a keratinase-like enzyme. In the former medium, not only the culture of the organism but also the purification procedures are easily performed. A higher production of proteolytic activity will be attained when more suitable cultural conditions are found for the non-keratinic medium.

NOVAL AND NICKERSON[1] found that the addition of glucose or casein hydrolyzate to the wool–salt medium retarded the digestion of wool by *S. fradiae* in spite of the greater growth, which indicated that the native keratin played an important role as if it were an inducer. However, our present study showed that a keratinase or a keratinase-like enzyme was produced in the absence of keratin material, which might refute the above speculation, NICKERSON, NOVAL AND ROBISON[2] have further found that the keratinolytic enzyme did not cause any release of sulfhydryl-containing peptides from wool, while such sulfhydryl-containing substance was accumulated[1] in the fermentation broth of keratin–salt medium by *S. fradiae*. It is well known that wool that has been reduced by alkaline thioglycolate (*i.e.* "Kerateine") is readily digested by common proteolytic enzymes. We therefore consider that digestion of wool by *S. fradiae* initially occurs by the enzyme attacking the S–S bond of keratin and the formation of this enzyme is induced by addition of native keratin. The keratinolytic enzyme together with the multiple proteolytic enzymes then attack the reduced keratin.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Merck and Co Ltd. of U.S.A. for supplying keratinase concentrate "M-zyme". Thanks are also due to Drs. H. NISHIMURA and M. MAYAMA of this laboratory for supplying many strains of Streptomyces.

REFERENCES

1 J. J. NOVAL AND W. J. NICKERSON, *J. Bacteriol.*, 77 (1959) 251.
2 W. J. NICKERSON, J. J. NOVAL AND R. S. ROBISON, *Biochim. Biophys. Acta*, 77 (1963) 73.
3 W. J. NICKERSON AND S. C. DURAND, *Biochim. Biophys. Acta*, 77 (1963) 87.
4 K. MATSUSHIMA, *Nippon Nogeikagaku Kaishi*, 23 (1959) 116.
5 K. MORIHARA, *Biochim. Biophys. Acta*, 73 (1963) 113.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 S. SØRENSEN, *Biochem. Z.*, 7 (1907) 45.
8 R. WILLSTÄTTER AND E. WALDSCHMIDT-LEITZ, *Ber.*, 54 (1921) 2988.
9 R. WILLSTÄTTER, *Z. Physiol. Chem.*, 142 (1925) 245.
10 E. J. CONWAY, *Micro-diffusion Analysis and Volumetric Error*, Crosby Lockwood, London, 1950.
11 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.

12 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
13 A. HIRAMATSU AND T. OUCHI, *J. Biochem. Tokyo*, 46 (1959) 653.
14 Y. NARAHASHI AND M. YANAGITA, *Symp. Enzyme Chem. Japan*, 17 (1965) 285.
15 K. MIZUSAWA, E. ICHISHIMA AND F. YOSHIDA, *Agr. Biol. Chem. Tokyo*, 28 (1964) 884.
16 U. J. LEWIS, D. E. WILLIAMS AND N. G. BLINK, *J. Biol. Chem.*, 222 (1956) 705.
17 I. MANDL, S. KELLER AND B. COHEN, *Proc. Soc. Exptl. Biol. Med.*, 109 (1962) 923.
18 K. MORIHARA, H. TSUZUKI, T. OKA, H. INOUE AND M. EBATA, *J. Biol. Chem.*, 240 (1965) 3295.
19 I. MANDL AND B. B. COHEN, *Arch. Biochem. Biophys.*, 91 (1960) 47.
20 T. OUCHI, *Agr. Biol. Chem., Tokyo*, 26 (1962) 723.
21 K. MORIHARA AND H. TSUZUKI, *Ann. Rept. Shionogi Res. Lab.*, 13 (1963) 91.
22 M. A. STAHMANN, J. S. FRUTON AND M. BERGMANN, *J. Biol. Chem.*, 164 (1946) 753.