The second $CHCl_3$ eluant gave 120 mg of the dienone (IX) as colorless prisms, mp 167—168° (from benzene–hexane), the spectroscopic data of which were identical with the authentic sample.[3b]

Reduction of the Dienone (IX) with Lithium Aluminum Hydride——A mixture of 0.1 g of dienone (IX) and 50 mg of $LiAlH_4$ in 30 ml of dry THF was refluxed for 1.5 hr. After evaporation of the solvent, the residue was carefully made basic with 5% NH_4OH and then extracted with $CHCl_3$. The extract was washed with water, dried over Na_2SO_4, and evaporated to dryness. Chromatography of the product on 3 g of silica gel using $CHCl_3$–MeOH (99 :1) as the eluant gave 50 mg of enone (XI) as colorless prisms (from benzene–hexane), mp 141—142°. *Anal.* Calcd. for $C_{19}H_{23}O_3N$: C, 72.82; H, 7.40. Found: C, 72.79; H, 7.35. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1645. Mass Spectrum *m/e*: 313 (M^+), 256. NMR ($CDCl_3$) τ: 3.12, 3.30, 3.97 (3H, each s, two aromatic and one olefinic protons), 6.10 (3H, s, OCH_3), 6.14 (3H, s, OCH_3), 7.62 (3H, s, NCH_3).

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A New Semi-alkaline Proteinase produced by *Streptomyces cinereoruber*[1]

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Semi-alkaline proteinases having optimum pH between 8 and 9 of *Streptomyces* origin have been reported as products of *Streptomyces erythreus*,[3] *Streptomyces fradiae*[4] and *Streptomyces madurae*.[5] The proteolytic activity of all of the above semi-alkaline proteinases can be inactivated by addition of diisopropylfluorophosphate. We have recently isolated a new semi-alkaline proteinase from the cultured-broth of *Streptomyces* H 55- SY 7 which was isolated from a soil sample collected at Kochi prefecture and identified as *Streptomyces cinereoruber*.[6] The proteolytic activity of the new semi-alkaline proteinase is not inactivated by addition of diisopropylfluorophosphate, but inactivated by addition of ethylenediaminetetraacetate.

Production, isolation, purification and enzymatic characteristics of the proteinase are presented in this paper.

The semi-alkaline proteinase was produced by cultivation of *Streptomyces* H 55-SY 7 in shaking flasks containing a medium composed of starch, glucose, soy bean meal, yeast extract and various inorganic salts on a reciprocal shaker. The proteinase was precipitated from the broth filtrate by saturation with ammonium sulfate and the crude enzyme mixture was reprecipitated by addition of cold acetone after dialysis against water. The enzyme was purified by gradient column chromatography on DEAE-cellulose after gel filtration

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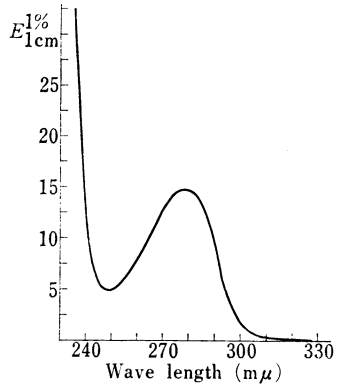

Fig. 1. Ultraviolet Absorption Spectrum in H_2O

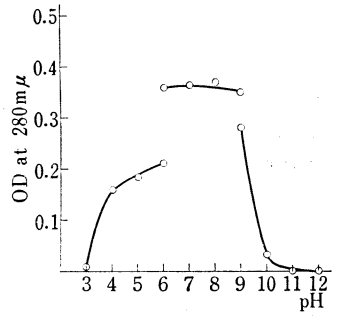

Fig. 3. pH Stability

1/10M Citrate buffer at pH 3—6, 1/10M Tris-HCl buffer at pH 6—9 and 1/20M borate buffer at pH 9—12 were used. A mixture of 0.1 ml of the aqueous enzyme solution (80 μg/ml) and 0.2 ml of the buffer was kept at room temperature for 1 hr. After adjusting the pH value to 8.0 and the total volume to 1 ml, the mixture was incubated with 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (pH 8.0) for 20 min at 37°.

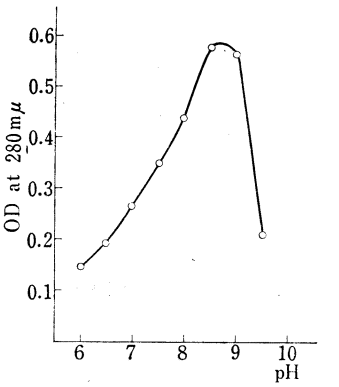

Fig. 2. Effect of pH on Proteolytic Activity

One ml of the aqueous enzyme solution (8 μg/ml) was incubated with 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (pH 6.0—9.5) for 20 min at 37°.

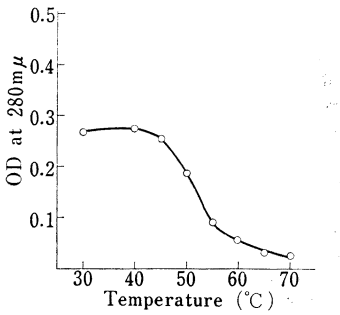

Fig. 4. Effect of Temperature on Proteolytic Activity

A mixture of 1 ml of the aqueous enzyme solution (4 μg/ml) and 1% casein solution in 1/10M Tris-HCl buffer (pH 8.0) was incubated at the test temperatures for 20 min.

on a column of Sephadex G 75. Gradient chromatography on CM-cellulose and following gel filtration on Sephadex G 75 were applied to obtain the most purified enzyme. The most purified proteinase showed one reddish spot at 13 mm toward the cathode by treatment with Ponceau 3R after electrophoresis at 1 mA/cm and 100 V on a cellulose acetate film for 1 hr using 0.1 M Tris-HCl buffer (pH 7.0).

The proteinase shows the ultraviolet absorption maximum at 278 mμ ($E^{1cm}_{1\%}$=11.95) in water as shown in Fig. 1. The optimum pH of the enzyme is around 8.5—9 when incubated with casein as seen in Fig. 2. The proteinase is stable at pH 6—9 when kept at room temperature for 1 hr, but the activity is almost lost at pH 3 or higher than pH 10 under the same

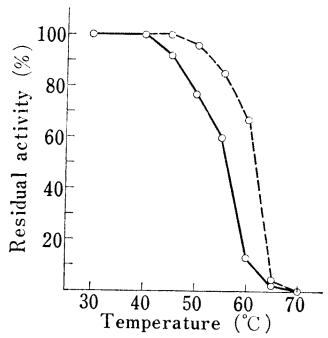

Fig. 5. Thermal Stability

One ml of the enzyme dissolved in 1/100M Tris-HCl buffer (pH 8.0) (8 μg/ml) was heated at the test temperatures for 10 min. After cooling, the resulting solution was incubated with 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (pH 8.0) for 20 min at 37°.
Added 1/1000M $(AcO)_2Ca$: ○-------○

condition as illustrated in Fig. 3. The effect of temperature on the proteolytic activity of the enzyme is shown in Fig. 4. The proteinase shows rather low optimum temperature at 30—40° when incubated with casein at pH 8.0. The proteinase is heated for 10 min at various temperatures with or without addition of 1/1000 M $(AcO)_2Ca$ as shown in Fig. 5 and addition of calcium ion shows a protective action for the proteolytic activity of the enzyme. The effects of various metal ions and inhibitors at a concentration of 1/1000 M on the proteolytic activity of the enzyme are shown in Table I. Mercuric or cupric ion inactivates most of the activity of the enzyme. The proteolytic activity of the enzyme is fully retained after addition of ω-chloroacetophenone, *p*-chloromercuribenzoate, diisopropylfluorophosphate or potato trypsin inhibitor, but lost entirely after addition of ethylenediaminetetraacetate or 8-hydroxyquinoline. Substrate specificities of the semi-alkaline proteinase are studied using amino

Table I. Effect of Various Inhibitors on Proteolytic Activity

Inhibitors	Residual activity (%)	Inhibitors	Residual activity (%)
$AgNO_3$	88	L-Cystine	79
$CaCl_2$	96	Ethylenediaminetetraacetate	0
$CoCl_2$	43	8-Hydroxyquinoline	0
$CuSO_4$	5	Diisopropylfluorophosphate	100
$FeSO_4$	78	Sodium laurylsulfate	88
$HgCl_2$	15	Iodine	0
Li_2SO_4	100	Glutathione (H)	100
$MgCl_2$	74	N-Bromosuccinimide	4
$MnCl_2$	65	Cyanogen bromide	85
$NaNO_2$	100	Hydroxylamine hydrochloride	100
$ZnSO_4$	31	Potato trypsin inhibitor[a)]	100
ω-Chloroacetophenone	100	Potassium permanganate	0
p-Chloromercuribenzoate	100		
Monoiodoacetic acid	81		

A mixture of 0.9 ml of the enzyme dissolved in 1/100M Tris-HCl buffer (pH 8.0) (9 μg/ml) and 0.1 ml of 1/100M inhibitor solution was kept for 20 min at rooom temperature. The mixture was incubated with 1 ml of 1% casein solution in 1/10M Tris-HCl buffer (pH 8.0) for 20 min at 37°.

a) 0.1 ml of a solution (300 μg/ml) was used.

Table II. Substrate Specificities

Substrates		Substrates	
D,L-Ala-Gly-Gly	—	N-Ac-Tyr-OEt	—
D,L-Ala-Leu	—	Ac-D,L-Val	—
Gly-Gly	—	Cbz-Glu-Phe	—
Gly-Leu	—	Cbz-Glu-Tyr	—
Gly-Phe	—	Cbz-Gly-Leu	—
Gly-Phe-NH_2	—	Cbz-Gly-Phe	—
Ac-Gly	—	Cbz-Gly-Phe-NH_2	+
Ac-D,L-Met	—	Cbz-Gly-Pro-Leu	±
N-Ac-Try	—	Cbz-Gly-Pro-Leu-Gly	—
N-Ac-Try-OEt	—	Cbz-Phe-Tyr	—
		Cbz-Try-Leu-NH_2	—

All amino acids are L-form, unless otherwise specified.

A mixture of 1 ml of the aqueous enzyme solution (8 μg/ml) and 1 ml of 1/400M substrate solution in 1/100M Tris-HCl buffer (pH 8.0) was incubated for 20 hr at 37° and lyophilized. The residue was dissolved in 0.1 ml of 50% aqueous methanol and chromatographed on silica gel G using BuOH: AcOH:H_2O=4:2:1. The digested substrate was detected by spraying 1% ninhydrin solution.

+: hydrolyzed, ±: weakly hydrolyzed, −: not hydrolyzed

acid derivatives and synthetic peptides as seen in Table II. The enzyme hydrolyzes carbobenzoxyl-glycyl-L-phenylalanine amide to give L-phenylalanine amide.

Thus, the semi-alkaline proteinase produced by *Streptomyces* H 55-SY 7 can be differentiated from the known semi-alkaline proteinases of *Streptomyces* origin as above mentioned.[3-5]

Experimental

Assay of the Proteolytic Activity——The modified casein-280 mμ method was used to determine the proteolytic activity.[7]

Production of the Semi-alkaline Proteinase——The inoculation seed was cultured in 500 ml shaking flasks containing each 100 ml of a medium composed of 0.5% malt extract, 0.5% glucose, 1.0% yeast extract and 0.3% NaCl (pH 7.2) on a reciprocal shaker (130 rpm) for 48 hr at 29°. A medium composed of 1.5% starch, 1.0% glucose, 2.0% soy bean meal, 0.5% yeast extract, 0.25% NaCl, 0.3% $CaCO_3$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $FeSO_4 \cdot 7H_2O$ (pH 7.6) was used for production of the semi-alkaline proteinase. The cultured seed (5 ml) was inoculated to each 140 ml of the production medium in 500 ml shaking flasks and shake-cultured on a reciprocal shaker for 70 hr at 29°.

Isolation of the Crude Semi-alkaline Proteinase——The cultured broth was filtered to remove the mycelium cake and the crude semi-alkaline proteinse was precipitated from the broth filtrate (7.7 liters, pH 8.4) by addition of $(NH_4)_2SO_4$ (4.5 kg) at 0° adjusting to pH 6.8. After the mixture was kept at 0° for 1 hr, the precipitate was collected by centrifugation and the precipitate dissolved in H_2O was dialyzed in a cellophane tube against distilled water for 5 hr. Cold acetone (540 ml) was added to the dialyzed solution (180 ml) and the precipitate collected by centrifugation was dried *in vacuo* to give the crude enzyme mixture (17.6 g).

Purification of the Semi-alkaline Proteinase——The crude enzyme mixture (8.65 g) dissolved in H_2O (10 ml) was dialyzed against distilled water for 3 hr and the dialyzate was purified by gel filtration on a column of Sephadex G 75 (45 cm × 3 cm diam.) eluted with H_2O. The eluate was collected in 15 ml of fractions and fractions 14—21 were lyophilized to give 416 mg of the residue having the proteolytic activity. The residue (416 mg) dissolved in H_2O (60 ml) was applied to a column of DEAE-cellulose (40 cm × 2 cm diam.) (Cl^--type treated with 0.1M Tris-HCl buffer of pH 7.0). The column was eluted with a linear gradient of aqueous NaCl at a concentration from 0 to 0.5M (total 600 ml) and the eluate was collected in 9.9 ml fractions. The semi-alkaline proteinase (86 mg) was recovered from fractions 6—11 by lyophilization after dialysis. The semi-alkaline proteinase above obtained (86 mg) dissolved in H_2O (40 ml) was further purified on a column of CM-cellulose (15 cm × 1.5 cm diam.) (NH_4^+-type treated with 0.1M Tris-HCl buffer of pH 7.0) eluted with a linear gradient of aqueous NaCl from 0 to 0.5M (total 600 ml). The eluate was fractionated to each 9.2 ml fraction and the fractions 3—8 were lyophilized after dialysis to give the semi-alkaline proteinase (66 mg). The semi-alkaline proteinase (66 mg) dissolved in H_2O (3.5 ml) was purified on a column of Sephadex G 75 (87 cm × 1.5 cm diam.) eluted with H_2O and the eluate was collected in 8.5 ml of fractions. The most purified semi-alkaline proteinase (19.8 mg) was recovered from fractions 7—12 by lyophilization.

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