Studies on Proteolytic Enzymes of *Streptomyces griseus*

Part I. Separation and Purification of Peptidase and Proteinase from Culture Media of *Streptomyces griseus* ATCC 3463*

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Separation and purification of proteolytic enzymes of *Streptomyces griseus* ATCC 3463 were undertaken by fractional precipitation with ethanol and ammonium sulfate followed by dialysis and finally applying zone electrophoresis on starch. In the experiment, peptidase free from proteinase and the latter enzyme free from the former were obtained. The purity was increased approximately 90-fold with the peptidase and 8-fold with the proteinase.

Nomoto and his coworkers^{1,2)} demonstrated the presence of a neutral protease in the culture filtrate of a strain of *Streptomyces griseus*. The enzyme digested various substrates for both endopeptidase and exopeptidase.

Tytell³⁾ has also purified a neutral proteinase, which acts on substrates commonly used for pepsin assay, from the culture media of a strain of *Streptomyces proteolyticus*. Up to date, few literatures have been published on peptidase of *Streptomyces* species. The present author has found that *Streptomyces griseus* ATCC 3463 showed the most potent peptidase- and proteinase activity among eight strains of *Streptomyces* species tested. In order to know more about the proteolytic enzymes of the strain, the proteolytic enzyme of the culture filtrate was purified and the above two enzyme activities were found to be ascribed to the two enzymes, proteinase and

peptidase, respectively. The present study deals with the method of separation of the proteinase and the peptidase and of purification of the two enzymes to make possible further study of their specificities. Studies of enzymatic properties of them will be presented elsewhere.

EXPERIMENTAL

Cultural Condition.

Streptomyces griseus ATCC 3463 was grown in Buillon medium containing one per cent glucose (pH 7.0) for three days at 27°C on a shaker. The culture filtrate was used as a crude enzyme solution.

Fractional Precipitation with Alcohol.

The culture filtrate was found to hydrolyze several kinds of substrates that are used for peptidase and proteinase activity assay. With the purpose of separating peptidase and proteinase from each other, the procedure of fractional precipitation with ethanol and ammonium sulfate was applied to the culture filtrate, and the enzymes were finally purified by zone electrophoresis on starch. The culture filtrate was chilled below 0°C and equal volume of cold ethanol was added. After standing of ten minutes, the precipitate occurred was removed by centrifugation and

* A part of this report was presented at the annual meeting of the Agricultural Chemical Society of Japan, Tokyo, April 6, 1960.

1) M. Nomoto and Y. Narahashi, *J. Biochem.*, **46**, 839 (1959).

2) M. Nomoto and Y. Narahashi, *ibid.*, **46**, 1481 (1959).

3) A.A. Tytell, J. Charney, W.A. Bolhofer and C. Curran, *Fed. Proc.*, **13**, 312 (1954).

the supernatant liquid was again chilled below 0°C. The ethanol concentration was then raised up to 66 per cent and after centrifugation, it was further increased to 80 per cent by the addition of cold ethanol. The precipitates at each ethanol concentration were collected by centrifugation and washed with cold ethanol and dried in vacuo. The precipitate obtained between zero and 50 per cent ethanol concentration showed a negligibly slight activity of peptidase and proteinase. The most active proteinase activity was found in the fraction obtained between 50 and 66 per cent ethanol concentration. On the other hand, the precipitate that occurred between 66 and 80 per cent ethanol concentration was remarkable in its peptidase activity. The two fractions were subjected to further purification.

Purification of Peptidase.

Two grams of the dried powder was extracted twice with 15 ml portions of water at room temperature and then the solution was centrifuged (3000 r.p.m., fifteen min.). To the supernatant solution (30 ml) was added solid ammonium sulfate to 0.4 saturation at pH 7.0 and 0°C, and after one hour standing the precipitate formed was removed by centrifugation (12000 r.p.m., fifteen min.). An additional solid ammonium sulfate was added to make 0.8 saturation at pH 7.0 and then the solution was left to stand for one hour followed by centrifugation as before. The precipitate was collected, dissolved in 10 ml of water, and dialyzed against water at pH 8.0 below 2°C. To the dialysate was again added solid ammonium sulfate to 0.5 saturation at pH 7.0, then the solution was allowed to stand in the cold for one hour and centrifuged (12000 r.p.m., fifteen min.). To the supernatant solution, further solid ammonium sulfate was added to make 0.7 saturation at pH 7.0 and after standing of one hour the solution was centrifuged as before. The precipitate thus obtained was collected, dissolved in 2 ml of cold water, and dialyzed against water changed every one hour at pH 8.0 below 2°C.

The dialyzed solution was then transferred into a short test tube and heated at 60°C for twenty minutes in order to remove contaminated other proteolytic enzymes. Then the solution was quickly chilled in an ice water bath at 0°C, and centrifuged to remove the precipitate formed by heating.

The final step in the purification was performed

by use of a zone electrophoresis apparatus (Model 2 of Tōyō Roshi Co., Ltd., Japan) in a cold room at around 3°C. After electrophoresis the enzyme was eluted from the starch columns cut into 1-cm-length.

Purification of Proteinase.*

The purification of the proteinase was carried out by a method similar to that applied to the peptidase. The dried powder obtained between 50 and 66 per cent ethanol concentration was extracted twice with water at room temperature and a clear solution was obtained by filtration. To the solution was added solid ammonium sulfate and the precipitate obtained between 0.3 and 0.5 saturation of the salt was collected, dialyzed and subjected to the electrophoresis.

Preparation of Substrate.

DL-Leucylglycine** was synthesized according to the Fischer's method⁴. Glycylglycine, glycylglycyl-L-leucine were synthesized by the phthalyl method⁵. DL-Leucylglycylglycine was synthesized by means of both the above methods. L-Leucine amide was prepared after Smith's method⁶. Casein was prepared from fresh milk according to the usual method⁷.

Determination of Peptidase Activity.

Peptidase activity was assayed by the alcohol titration method of Grassmann and Heyde as reported previously^{8,9}. Unless otherwise noted, the condition of digestion was as follows: 0.05 M tris pH 8.0; 0.10 M Leu-Gly-Gly; total volume of digestion mixture, one ml; digestion period, one hour at 40°C. Hydrolysis is expressed as 100 per cent for complete splitting of one peptide bond. Activity is expressed as the zero order constant k_0 . Specific activity C_0 was defined as $C_0 = k_0/E$, where E is the enzyme protein N in mg per ml of the reaction mixture. The total activity in units was calculated from the C_0 value being multiplied by number of mg of protein N contained

* Another method for purification of the enzyme is now being studied using Duolite C-10 ion exchanger column.

** The following abbreviations are used: Gly-Gly, glycylglycine; Gly-Gly-Leu, glycylglycyl-L-leucine; Leu-Gly, DL-leucylglycine; Leu-Gly-Gly, DL-leucylglycylglycine; Leu-NH₂, L-leucine amide; tris, tris (hydroxymethyl) aminomethane; TCA, trichloroacetic acid.

4) E. Fischer, "Untersuchungen über Aminosäuren, Polypeptide und Proteine", Verlag von Julius Springer, Berlin, I (1906), II (1919).

5) J.C. Sheehan and V.S. Frank, *J. Am. Chem. Soc.*, **71**, 1856 (1946).

6) E.L. Smith and N.B. Slonim, *J. Biol. Chem.*, **176**, 835 (1948).

7) E.J. Cohn and J.L. Hendry, "Organic Syntheses" (in Japanese), Maruzen, 1951, p. 157.

8) W. Grassmann and W. Heyde, *Z. Physiol. Chem.*, **183**, 32 (1929).

9) M. Sato, T. Ouchi, A. Hiramatsu and T. Akatsuka, *Ibaraki Daigaku Nōgakubu Gakujutsu Hōkoku*, **4**, 59 (1956); M. Sato and T. Akatsuka, *This Journal*, **23**, 465 (1959).

in enzyme solution.

Determination of Proteinase Activity.

Proteolytic activity was measured according to the method of Anson^{10,11}. The incubation system consisted of one ml of 0.6 per cent casein in 0.05 N $\text{NH}_4\text{OH-NH}_4\text{Cl}$ buffer (pH 10.0), and 0.2 ml of enzyme solution, and the mixture was placed in a small test tube with two limbs. The substrate solution was pipetted into one of the limbs and the enzyme solution into the other. The vessel was first kept for five minutes at 40°C, and then the two solutions were mixed. The mixture was incubated at 40°C for thirty minutes. At the end of incubation, one ml of 1.8 per cent trichloroacetic acid was added to the mixture and the resulted precipitate was removed by filtration after standing for thirty minutes at 40°C. In the control system the reaction mixture was incubated without the enzyme solution which was added immediately after the addition of trichloroacetic acid. The extent of hydrolysis during the incubation period was determined as follows: To one ml of the filtrate 2.5 ml of 0.55 M sodium bicarbonate was added, followed by 0.5 ml of three fold diluted Folin's reagent¹². After thoroughly mixing of the solution and standing for thirty minutes at 30°C, the blue color developed was measured by use of an electric photometer (Model 2 of Tōyō Roshi Co., Ltd., Japan) at 660 m μ .

The enzyme activity is expressed in units, one unit being defined as the amount of enzyme that liberates TCA soluble hydrolyzate corresponding to one μ mole as tyrosine per minute under the condition. Specific activity was expressed in units per mg of protein N of the enzyme; the total activity in units of an enzyme solution was calculated from the specific activity multiplied by mg of protein N in the enzyme solution.

Protein Determination.

The amount of protein was determined by either the micro-Kjeldahl method or Lowry's method¹³.

RESULTS AND DISCUSSION

Among 8 strains of *Streptomyces** which

seem to have a comparatively strong proteolytic enzyme, 4 strains such as *Streptomyces lavendulae* ATCC 8664, *Streptomyces griseus* ATCC 3463, *Streptomyces griseus* ATCC 10137 and *Streptomyces griseus* X-28 showed strong activities of peptidase and proteinase.

Of these 4 *Streptomyces* strains, *Streptomyces griseus* ATCC 3463 had the most potent peptidase- and proteinase activity. As for the action of peptidase of the fungus, the filtrate of the cultural media showed a strong hydrolytic action on leucyl peptide such as Leu-Gly-Gly, Leu-Gly and Leu-NH₂. But it does not act on Gly-Gly and Gly-Gly-Leu. In addition to the peptidase activity mentioned above, the filtrate also showed a strong proteolytic action for casein. From the culture filtrate of this fungus, peptidase was fractionally precipitated at 66 to 80 per cent ethanol concentration followed by salting out at 0.5 to 0.7 saturation with ammonium sulfate at pH 7.0, but proteinase was precipitated between 50 and 66 per cent ethanol concentration and salted out with ammonium sulfate from 0.3 to 0.5 saturation at pH 7.0. Each concentrated enzyme was then dialyzed and subjected to zone electrophoresis on potato starch. Application of this process presented here enabled the author to separate and purify the two enzymes from the culture filtrate of the fungus (Table I, Fig. 1 and 3). Fig. 2 shows a migration pattern of peptidase activity in the second run, indicating that the enzyme is electrophoretically a single enzyme protein. It was indicated that the activity was in accordance with the protein peak. Fig. 4 shows the time course of the peptidase reaction. By this procedure, the peptidase was isolated in a state completely free of proteinase. The proteinase was also purified without any contamination of peptidase (Fig. 3). Good reproducibility was obtained by the method mentioned above in the purification

10) M.L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938).

11) S. Akabori, "Method in Enzymology", (in Japanese) II, Asakura press, 1957, p. 242.

12) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

13) O.H. Lowry, N.J. Rosenbrough, A.L. Forr and R.T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

* Other strains tested are as follows: *Streptomyces antibioticus* NRRL-B 546, *Streptomyces reticuli* IFO 3354, *Streptomyces griseolus* ATCC 11796 and *Streptomyces retgersensis* WK 3350.

TABLE I. PROCEDURES FOR PURIFICATION OF PROTEINASE AND PEPTIDASE

Step	Proteinase	Peptidase
Crude solution	Concentrated to 1/2 vol.	Concentrated to 1/2 vol.
Ethanol precipitation	50~66%	66~80%
1st ammonium sulfate precipitation	0.3~0.5 saturation	0.4~0.8 saturation
1st dialysis at	pH 8.0	pH 8.0
2nd ammonium sulfate precipitation	—	0.5~0.7 saturation
2nd dialysis at	—	pH 8.0
Heating	—	60°C, 20 min.
1st electrophoretic separation*	10 cm	5 cm
2nd electrophoretic separation*	—	5 cm

* Distance of migration toward cathode from point of application on starch block.

FIG. 1. Distribution of Enzyme Activities and Protein N along the Starch Zone by the Electrophoretic Run.

The first run was performed at 6°C for 15 hours at 14 m amp and 400 V. The buffer used was 0.06 M veronal pH 8.6. The enzyme moved toward the cathode. After the run, the starch block was cut into 1-cm-length and eluted with 5 ml of water, the eluate were assayed for protein N and the proteolytic enzyme activity.

---○--- Peptidase
—●— Proteinase
—○— Protein N

and separation of both peptidase and proteinase. The change in activity during purification of peptidase and proteinase are shown in Table II and III, respectively. The purity of peptidase was found to increase to about 90-fold, and proteinase approximately to 8 times.

FIG. 2. Separation of Peptidase by the Zone Electrophoresis on Potato Starch.

The second run was performed at 6°C for 19 hours at 14 m amp and 320 V. The buffer used was 0.06 M veronal pH 8.6. The enzyme moved toward the cathode. The assay method of the proteolytic enzyme was the same as those in Fig. 1.

---○--- Peptidase
—●— Proteinase
—○— Protein N

FIG. 3. Movement of Proteins and Proteinase by the Zone Electrophoresis on Potato Starch.

The run was performed at 6°C for 20 hours at 7.5 m amp and 210 V. The buffer used was 0.06 M veronal pH 8.6. The enzyme moved toward the cathode. The assay method of the proteolytic enzyme was the same as those in Fig. 1.

---○--- Peptidase
—●— Proteinase
—○— Protein N

In an experiment with a strain of *Streptomyces griseus* applying the column chromatography with Kaken C-1, Nomoto and his coworkers^{1,2)} succeeded in the purification to obtain a single component protease which

hydrolyzed various substrates for both endo-

FIG. 4. The Hydrolysis of Leu-Gly by Peptidase.

The reaction mixture contained 0.5 ml of substrate buffer solution (0.20 M Leu-Gly in 0.10 M tris, pH 8.0), 0.1 ml of enzyme solution (0.2 μ g of protein N per ml) and water in a total volume of 1.0 ml. Incubated at 40°C. At intervals, 0.2 ml aliquots were withdrawn, and titrated with 1/100 N KOH ethanol solution.

TABLE II. PURIFICATION OF PEPTIDASE FROM CULTURE FILTRATE

Fraction	Total protein N mg	Total units	Specific activity
Crude solution	160	162880	1018
Ethanol precipitation	95.2	109480	1150
1st ammonium sulfate precipitation	41.7	51000	1223
2nd ammonium sulfate precipitation	7.7	49800	6468
Heating	6.6	53230	8065
1st electrophoretic separation	0.43	19435	48585
2nd electrophoretic separation	(46)*	4535	98590

* μ g.

This is a typical run for 2000 ml of culture media.

TABLE III. PURIFICATION OF PROTEINASE FROM CULTURE FILTRATE

Fraction	Total protein N mg	Total units	Specific activity
Crude solution	160	23500	145
Ethanol precipitation	30.6	5416	177
Ammonium sulfate precipitation	5.1	2478	486
Electrophoretic separation	0.3	263	876

This is a typical run for 2000 ml of culture media.

peptidase and exopeptidase assay. The proteolytic enzyme of *Streptomyces griseus* ATCC 3463 is quite different from the protease of *Streptomyces griseus* studied by Nomoto and his coworkers. The results described here show that the proteolytic enzyme of *Streptomyces griseus* ATCC 3463 consists of, at least, two kinds of enzymes, namely peptidase and proteinase. Further studies on properties of the both enzyme will be presented elsewhere and discussed.

SUMMARY

1. A method for isolating peptidase and proteinase from culture media of *Streptomyces griseus* was described.

2. It was shown that in the culture media of *Streptomyces griseus* ATCC 3463, peptidase and proteinase were present and the two enzymes were obtained in a highly purified state, respectively.

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