STUDIES ON PROTEOLYTIC ENZYMES OF *ASPERGILLUS WENTII*

II. Purification and Some Properties of the Proteolytic Enzymes of *Aspergillus wentii*

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Proteolytic enzymes are tools which have been used in medicine and industry for hundreds of years. However, in recent years the commercial uses of proteases have increased and will probably continue to increase as standard, active enzyme preparations with good solubility and odor become available. For this reason the preparation of a new protease from the micro-organisms is an important event which can benefit both industry and medicine.

From the native fermentation food, a kind of fungus which can produce a remarkable amount of proteolytic enzymes has been isolated by the authors (Su *et al.*, 1966): The taxonomical characteristics of this fungus were examined and it was found to belong to *Aspergillus wentii.* Studies on the various conditions for the production of proteolytic enzymes by this fungus were described in the previous paper. In this paper, a study of the purification and some physical and chemical properties of the proteolytic enzymes are presented.

Materials and Methods

Preparation of Crude Enzyme.

The medium which contained defatted soybean powder 2.5%, rice bran 0.5%, sucrose 0.5% and calcium carbonate 0.3% was prepared and sterilized at 120°C for fifteen minutes. A strain of *Aspergillus wentii* was then inoculated on the sterilized medium. The optimal cultural conditions which were cultivated in a 5 *l*-jar fermentor (New Brunswick Scientific Co., Type model FS-605) were under temperature 35°C; initial pH 5.0; rate of agitation, 300 r. p. m.; and rate

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of aeration, 1/2 volume per volume of medium per minute. After seventy-five hours, maximum yield of protease was obtained and the cultural broth was centrifuged. Three liters of the supernatant was made 65% saturated with ethanol at 0°C. After being centrifuged, the precipitate there occurred was dried in vacuo and about 6 g of crude enzyme preparation was obtained.

Activity Assay of the Protease.

Proteolytic activity was determined at pH 7.5 and 30°C using casein (Hammersten, Merck) according to the modification by Hagiwara of Anson's method (Hagiwara, 1954) as has been reported in the previous paper. Proteolytic activity was represented as units [PU] by expressing micrograms of tyrosine liberated per minute in the hydrolysis of 0.6% casein at 30°C.

Activity Assay of Amylase.

Sumner's method (Sumner *et al.*, 1935) was employed for determination of the amylase activity. One ml of properly diluted enzyme solution was incubated with 1 ml of the substrate solution for definite time at 30°C. The enzyme reaction was interrupted by the addition of 2 ml of dinitrosalicylic acid reagent. The tube containing this mixture was heated for 5 minutes in boiling water and then cooled in running tap water. After addition of 20 ml of water, the optical density of the solution containing the brown reduction product was determined photometrically at 540 mμ and a blank was prepared in the same manner without enzyme. A calibration curve established with maltase was used to convert the colorimeter readings into miligrams of maltase.

Determination of Protein.

Phenol method (Lowry, 1951) was employed to determine the protein of enzyme solution. Specific activity of protease was expressed by proteolytic activity per 1 mg of protein.

Results and Discussion

pH-Stability of the Protease in the Crude Enzyme Preparation.

The crude enzyme preparation was dissolved in various buffer solutions (citrate-phosphate buffer, pH 2.2–5.1; phosphate buffer, pH 6.0–8.0; glycine-NaOH buffer, pH 9.3–11.2), and the solution was incubated at 30°C for thirty minutes. After incubation, each enzyme solution was added to the substrate to determine the activity of protease.

As shown in Table 1, the protease was stable at pHs between 7.0 and 9.3. Thus, the enzyme solution was adjusted within the range of pHs from 7.0 to 9.3 during whole course of purification.

Removal of Amylase by Adsorption on Corn Starch in Alcoholic Solution.

In order to remove the amylase contained in crude enzyme, Sawada's method (Sawada, 1963) was applied.

Table 1. *Effect of pH on the Stability of Protease**

pH at incubation	Residual activity (%)
Before incubation	100
2.2	0
3.1	0
4.1	0
5.1	10.65
6.0	48.65
7.0	97.00
7.5	99.00
8.0	98.00
9.3	97.50
10.1	86.60
11.2	60.00

* One hundred mg of crude enzyme preparation was dissolved in 100 ml of water and 0.5 ml of enzyme solution was mixed with 2 ml of buffer of various pH. After incubation at 30°C for 30 min., 0.5 ml of the mixture was used to determine residual activity at pH 7.5.

The crude enzyme preparation was dissolved in water, then centrifuged. The supernatant was adjusted to pH 7.0 and made 40% saturated with ethanol. Then corn starch was added to the alcoholic solution in a proportion of 200 g to 1 *l*. The mixture was stirred for two hours at the temperature below 5°C and centrifuged to remove the residue containing corn starch. The supernatant was used to determine the activity of amylase. As shown in Table 2, the activity of amylase which was contained in the crude enzyme was very weak.

Table 2. *Corn Starch Adsorption in Ethanol of 40% Concn.**

Reaction time (min.)	[PU]/mg protein	Yield of total activity of protease (%)		Activity of amylase [O. D.] at 540 mμ	
		Corn starch		Corn starch	
		Added	Not Added	Added	Not Added
20	550	56	100	0.215	0.710

* Crude enzyme preparation 100 mg was dissolved in 15 ml of water and centrifuged. Supernatant added ethanol in a 40% concentration and corn starch of 20% (w/v) against alcohol solution. Stirred for two hours at 5°C, centrifuged and determine the activity of amylase.

As there was little difference between both the residual ratio of the activity of protease and amylase, this procedure might not be a much effective method for the removal of amylase. But both the enzyme were hardly separated from each other, even by column chromatography using carboxymethylcellulose and by precipitation of either enzyme by combining with revanol. A modification

of this procedure which is being examined at present, so the problem of amylase was not considered through the whole course of purification.

Alcohol Fractionation.

The crude enzyme preparation was dissolved in M/10 phosphate buffer of pH 7.5, then centrifuged; the supernatant was fractionated with 65% ethanol at the temperature below 5°C. By this fractionation, the specific activity of protease was increased to 1.6 times as high as that of the crude enzyme preparation.

Calcium Acetate Treatment.

The precipitate obtained by second fractionation with 65% ethanol was dissolved in M/10 phosphate buffer of pH 7.5. After the enzyme solution was allowed to stand over-night at 0°C, the solution was centrifuged to remove the residue of impurities. Then, in order to remove impurities, 1M calcium acetate was added to the enzyme solution.

This procedure was not a very effective method to increase the specific activity of protease, but it could increase the heat stability of protease during the course of purification. On the other hand, the enzyme solution became clear particularly after the treatment.

Ammonium Sulfate Fractionation.

The supernatant obtained after the addition of 1M calcium acetate was dialyzed using cellophane membrane toward running tap water for one day, and the dialyzed enzyme solution was fractionated by 40% saturated ammonium sulfate at pH 7.5 and 5°C. Precipitate was removed and the supernatant was fractionated by 85% saturated ammonium sulfate. By this fractionation, the specific activity of protease was increased to 2 times as high as that of the crude enzyme preparation.

Sephadex G-25 Column Treatment.

The precipitate obtained by the addition of ammonium sulfate of 85% saturation was dissolved in M/10 phosphate buffer of pH 7.5, and the protease and ammonium sulfate contained in the solution were separated from each other by gel filtration using a column of cross linked dextran, Sephadex G-25, instead of the removal of the salt by dialysis (Nunokawa, 1963). A 3×40-cm Sephadex G-25 column was packed by gravity and equilibrated with the same phosphate buffer solution, and 20 ml of the enzyme solution was allowed to pass through the column at a rate of approximately 1 ml per minute. The results were shown in Fig. 1. The proteolytic activity appeared at the first 50 ml of effluent, and no ammonium sulfate was collected. On the other hand, the curve of proteolytic activity and the curve of absorbancy at 280 mμ were in agreement through the course of effluent.

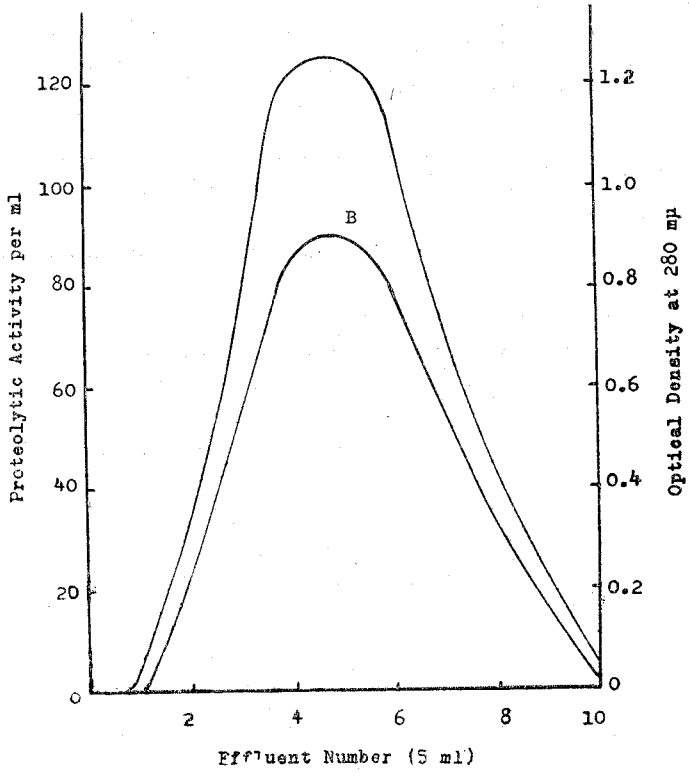

Fig. 1. Enzyme Treated by Sephadex G-25 Column
A: Absorbancy at 280 mμ
B: Proteolytic activity

Acetone Fractionation.

Three to seven fraction of Sephadex G-25 column effluent were collected and it was submitted for the fractionation of protease with 60% acetone below 0°C. The precipitate was dried in vacuo at a low temperature. The enzyme appeared transparent and in flake shape. By the fractionation, the specific activity of protease increased to about 2.3 times as hish as the crude enzyme preparation.

The purification procedure was shown in Diagram 1. The specific activity and yield of protease in each fraction were summarized in Table 3.

Diagam 1. Purification Procedure of the Protease

Cultural broth (3000 ml)

↓ Added ethanol in a 65% concn., centrifuged.

Crude enzyme preparation (6 g)

↓ Dissolved in M/10 phosphate buffer of pH 7.5, then added ethanol in a 65% concn., centrifuged.

Crude enzyme (3 g)

↓ Dissolved in M/10 phosphate buffer of pH 7.5 and added 1M calcium acetate, centrifuged.
↓ Dialyzed over-night, centrifuged.

Dialysate (70 ml)

↓ Added ammonium sulfate to make the final concentration of 0.4 sat., centrifuged.

Supernatant (76 ml)

↓ Added ammonium sulfate to make the final concentration of 0.85 sat., centrifuged.

Precipitate

↓ Dissolved in M/10 phosphate buffer of pH 7.5.
↓ Fractionated through Sephadex G–25 column.

Eluate of protease fraction (20 ml)

↓ Added acetone in a 60% concentration, centrifuged.

Enzyme precipitate (60 mg)

Table 3. *Specific Activity and Yield of Each Fraction in Purification of the Protease*

Fraction	Specific activity (PU/mg of protein)	Total activity (PU)	Recovery (%)
Crude enzyme preparation	400	132,000	100
Second 65% EtOH precipitate	650	90,750	69.4
1M Calcium acetate treatment	710	75,000	57.8
Dialysate	750	70,000	53.7
Precipitate in 0.85 sat. ammonium sulfate salting out	800	55,000	42.5
Treatment with Sephadex G-25 column	825	46,900	34.7
Precipitate with 60% acetone	925	34,650	26.3

After purification, the specific activity was increased to about 2.3 times as high as that of the original crude enzyme preparation, and the yield of total activity of protease was 26.3%.

Optimum pH of Protease.

The purified enzyme was dissolved in various buffer solutions (citrate-phosphate buffer, pH 3.0–5.0; phosphate buffer, pH 5.5–8.5; glycine-NaOH buffer, pH 9.0–11.0) and incubated at 30°C for thirty minutes. The proteolytic activity was determined at various pHs. The results were shown in Fig. 2. At pH 6.6 and pH 10.0, it showed having two high activity peaks. So that the purified enzyme might contain two kinds of enzyme.

Matsushima (1958) has reported that three kinds of protease, acid-, neutral-, and alkaline-protease, are found in fungal extract. These proteases from various fungi have been studied by many investigators (Nunokawa, 1962; Jönsson *et al.*, 1964, 1965; Kishida *et al.*, 1966; Sawada, 1966). It was found

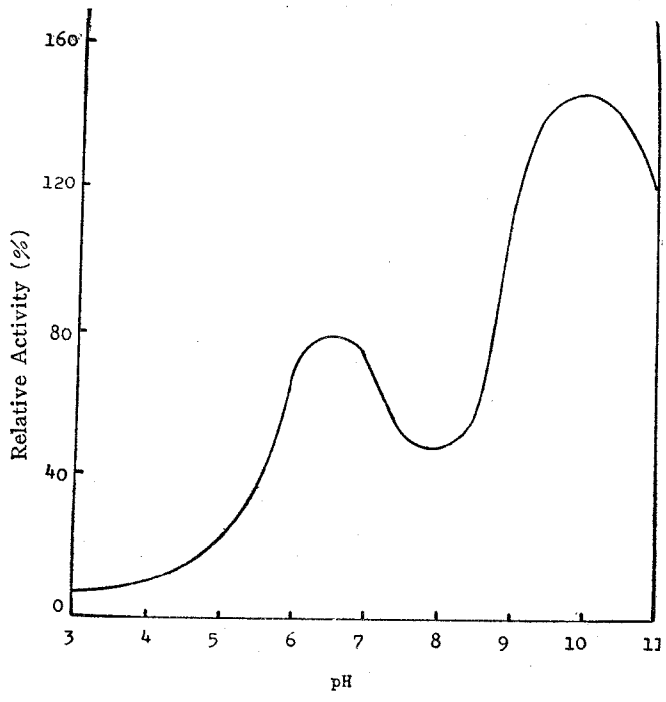

Fig. 2. Optimum pH for Proteolytic Activity

that some fungi were proved to produce different types of proteases simultaneously, and according to the major components of their protease systems, fungi could be divided into four types: *Aspergillus oryzae* type, *Aspergillus niger* type, *Penicillum leutem* type and *Hormodendrum* sp. type. According to this division, the proteolytic enzymes produced by *Aspergillus wentii* belonged to *Aspergillus oryzae* type.

Effect of the Temperature on Protease Reaction.

Under various temperature, the purified enzyme reacted with substrate. The results were shown in Fig. 3. The optimal reaction temperature was at 40°C, and over 50°C, the protease activity decreased rapidly.

Heat and pH Stability of Protease.

The purified enzyme was dissolved in phosphate buffer of pH 6.6 and glycine-NaOH buffer of pH 10.0 respectively, incubated at 40°C for determining the heat and pH stability of protease. The results were shown in Fig. 4. The purified enzyme was more stable at pH 6.6 than pH 10.0. At pH 10.0 and 40°C, the proteolytic activity decreased rapidly.

Effect of Metallic Ions on Proteolytic Activity.

The purified enzyme was dissolved in the solution which contained 0.02 mM per ml of various metallic ions and determined the proteolytic activity. The results were shown in Table 4.

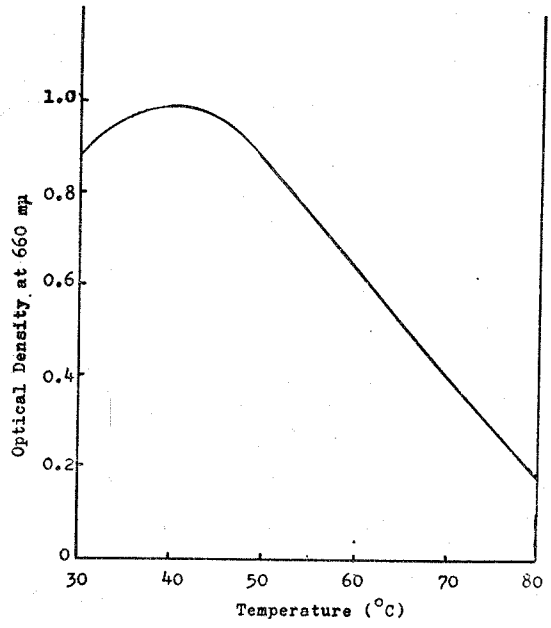

Fig. 3. Effect of Temperature on Proteolytic Activity
Enzyme concentration: 1 mg/ml

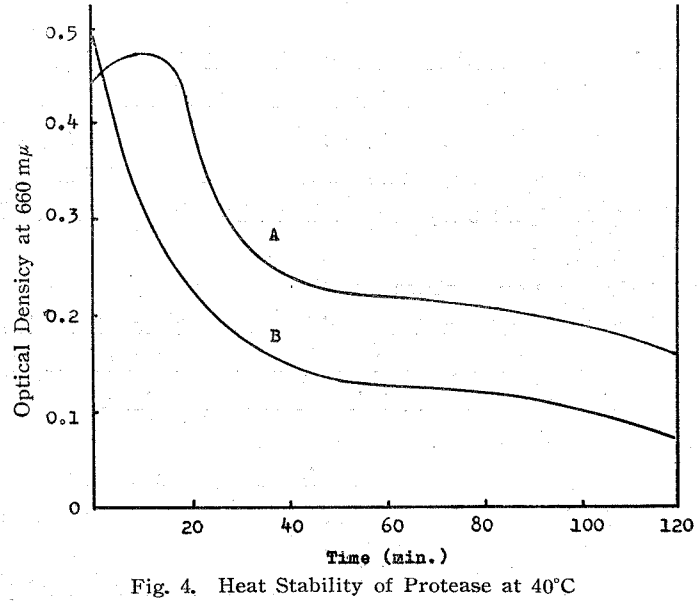

Fig. 4. Heat Stability of Protease at 40°C
Enzyme concentration: 0.5 mg/ml
A: pH 6.6 B: pH 10.0

From Table 4, it was found that Ca^{++} and Mn^{++} activated the enzyme activity. Fe^{++}, Zn^{++}, Co^{++}, Mg^{++} showed no effect and Cu^{++} inhibited the enzyme activity.

Table 4. *Effect of Various Metallic Ions on Proteolytic Activity*

Metallic Salt*	Relative Activity (%)**
Not added	100
Calcium carbonate	108.5
Ironous sulfate	94.5
Manganese chloride	108.5
Zinc sulfate	94.5
Cobaltous chloride	98.0
Copperic sulfate	56.5
Magnesium sulfate	96.5

* Salt concentration: 1 mM/50 ml
** Enzyme concentration: 1 mg/ml

Comparison of Various Substrates for Protease Reaction.

Various substrates with the same concentration were reacted with the purified enzyme to compare the ability of hydrolysis. The results were shown in Table 5. The relative hydrolysis of casein was higher than gelatin.

Table 5. *Comparison of Various Substrates for Protease Reaction*

Substrate added (0.6 %)	Acid hydrolysis* (PU/ml)	Enzyme hydrolysis** (PU/ml)	Relative hydrolysis*** (%)
Casein	112.0	52.9	47.2
Gelatin	260.0	28.6	11.0

* Acid hydrolysis: One volume of 0.6% substrate mixed with one volume of 12 N HCl and hydrolyzed at 110°C for 6 hours.
** Enzyme hydrolysis: Enzyme solution (1 mg/ml) reacted with 0.6% substrate at 30°C for 10 minutes.
*** Relative hydrolysis: $\frac{\text{Enzyme hydrolysis}}{\text{Acid hydrolysis}} \times 100$ (%)

Summary

1. When a strain of *Aspergillus wentii* was cultured on defatted soybean powder medium, the fungus produced two kinds of protease which was most active at pHs 6.6 and 10.0 respectively on casein as substrate. The enzyme was purified, after precipitation of the protease by adding ethanol to the cultural broth, removal of amylase by adsorption on corn starch in alcoholic solution, fractional precipitation of the protease with ethanol, calcium acetate treatment, and ammonium sulfate and acetone fractionation were performed in that order.

2. The purification procedure increased the specific activity of protease about 2.3 times as high as that of the original crude enzyme preparation, and the yield of total activity of protease was 26.3%.

3. The protease was optimally active at pHs 6.6 and 10.0, and 40°C toward casein as substrate. Ca^{++} and Mn^{++} activated the proteolytic activity whereas Cu^{++} inhibited it.

Aspergillus wentii 所分泌蛋白質分解酵素之研究

II. *Aspergillus wentii* 所分泌蛋白質分解酵素之精製與性質

蘇遠志 劉文雄 鄭篤誠

著者等從臺灣之醱酵食品中，分離出具有强力蛋白質分解酵素活性之菌株 *Aspergillus wentii*。其菌學性質及酵素之生產條件，著者等曾於第一報（中國農業化學會誌，1966 年英文特刊）提出報告。本實驗乃是將此酵素初步純化，以探討其理化學性質。茲將實驗結果之摘要說明如下：

1. 酵素之純化：酵素之純化步驟依次爲①第一次酒精沉澱②第二次酒精沉澱③加醋酸鈣④透析 ⑤硫銨沉澱 ⑥ Sephadex G-25 column 處理 ⑦丙酮沉澱。經上述處理後所得酵素之比活性爲 925 PU/mg of protein，與粗酵素之活性比較已提高 2.3 倍，其回收率爲粗酵素之26.3%。
2. 酵素之特性：以酪朊（casein）爲基質時，酵素之最適作用溫度爲 40°C，其最適作用 pH 爲 6.6 及 10.0。金屬離子 Mn^{++} 及 Ca^{++}對本酵素之活性有促進作用，而 Cu^{++}卻有抑制作用。

Literature Cited

HAGIWARA, B. A method for determination of proteolytic activity. Ann. Rep. Sci. Work, Osaka Univ., **2**: 35, 1954.

JÖNSSON, A. G., and S. M. MARTIN. Protease production by *Aspergillus fumigatus*. Agr. Biol. Chem., **28**: 734, 1964; Protease production by *Alternaria tenuissima*. ibid., **29**: 787, 1965.

KISHIDA, T., and S. YOSHIMURA. Studies on proteinases from *Gliocladium roseum*. Agr. Biol. Chem., **30**: 1183, 1966.

LOWRY, O. H. Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**: 265, 1951.

MATSUSHIMA, K. Studies on the proteolytic enzymes of molds, Part XIV. Classification of fungal protease systems by means of pH-activity curves. J. Agr. Chem. Soc. Japan, **32**: 215, 1958.

NUNOKAWA, Y. Studies on the constitution of "rice-koji" protease. Part II. The general properties of acid- and alkaline-protease. J. Agr. Chem. Soc. Japan, **36**: 879, 1962; Purification of the acid-proteases from the mold with Sephadex. J. Fermentn. Assoc. Japan, **21**: 379, 1963.

SAWADA, J. Studies on the acid-protease of *Paecilomyces varioti* Bainier TPR-220, Part I. Crystallization of the acid-protease of *Paecilomyces varioti* Bainier TPR-220. Agr. Biol. Chem., **27**: 677, 1963; Part IV. Physical and chemical properties of the crystalline acid-protease. ibid., **30**: 393, 1966.

SU, Y. C. and W. H. LIU. Studies on proteolytic enzymes of *Aspergillus wentii*, I. Identification of the organism and some cultural conditions for the production of proteolytic enzymes. Jour. of the Chin. Agr. Chem. Soc., Sp. Iss. 10, Dec., 1966.

SUMNER, J. B., and S. F. HOWELL. A method for determination of saccharase activity. J. Biol. Chem., **108**: 51, 1935.