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Purification and Characterization of Protease from *Sarcodon aspratus* (Berk.) S. Ito

능이버섯에서 분리한 Protease의 정제와 특성

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요 약

능이버섯 〔*Sarcodon aspratus*(Berk.) S.Ito〕 으로부터 단백질 가수분해 효소를 추출하여 75%$(NH_4)_2SO_4$ 염석과 DE52 anion exchange column chromatography 와 sepharyl-S 200 column 및 Mono s column chromatography 에 의해 정제하였는데 조 효소의 특이 활성은 55.2U/mg protein으로 조효소액에 비하여 11.26배 증가하였고 수율은 49.5%로 나타났다.

정제된 효소는 전기영동을 행한 결과 단일 band를 나타내었으며 분자량은 29,300으로 추정되었다.

pH의 안정성은 4℃에서 48시간 보존하였을 때 pH 8.5에서 가장 안정하였고, pH 5.5~10.5까지 높은 활성을 유지하였다.

온도에 대한 안정성은 30분간 보존 한 후 활성을 검토한 결과 단백분해능은 50℃까지 비교적 처음활성을 유지하다가 60℃에서는 53%정도의 활성이 유지되었으나 그 이상의 온도에서는 급격히 실화하여 70℃에서는 완전히 실화하였다.

금속 이온에 의해서는 크게 저해를 받지 않았으나 PMSF 저해제에 대하여 저해되어 본 효소가 serine protease임을 시사하였다.

Key words : *Sarcodon aspratus*, protease, enzyme, purification, activity

Ⅰ. Introduction

Mushroom has been related closely to our life and used as food as well as treatments and prevention to various diseases. Recently, as people get interested more in the health-improving effects of mushroom, the consumption of mushroom is increasing continuously as a non-polluting natural food and a functional food. Besides, edible mushrooms not only contain plenty of nutrients such as protein, vitamin, and minerals, but also have unique taste[1] and flavor[2,3]. Mushroom has been reported to have medical effects: anti-cancer[4,5] and antibiotic[6] action, control of various biologic functions, lowering blood cholesterol contents.

It is known that the enzymes produced by mushroom is almost hydrolase. Keishi et al.[7] reported that most of edible mushrooms have cellulase. Yamashki and Suzuki[8] separated and purified β-glucosidase and glacaomylase from *Lentinus edodes*. Gavrilova et al.[9] extracted protease from mushrooms and analyzed its catabolic ability for protein.

Eun et al.[10] analyzed the optimum pH, temperature, and substrate-specific property of metal ions to study the availability as a digestive.

While *Sarcodon aspratus* (Berk.) S. Ito, self-sown in Korea and Japan in late September and early October, has been popularly used long as the edibles and the medicinals, but not studied widely as other edible mushrooms are.

In the previous study of *Sarcodon aspratus* (Berk.) S. Ito, Park[11] verified that it has casein catabolic

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protease. Ryu[12] estimated that the main contributors of protease activity of *Sarcodon aspratus* (Berk.) S. Ito were trytophan and serine. Lee[14] from the study with the purpose of discovering the first structure of alkaline protease among *Sarcodon aspratus* (Berk.) S. Ito, revealed that it was Valine which are related to amino acid related to amino acid producing active parts and at the end of N. While Lee[14] and Eun et al.[15] studied on the medicinal action of *Sarcodon aspratus* (Berk.) S. Ito. Lee[16] compared the titer of protein catabolic ability of *Sarcodon aspratus* (Berk.) S. Ito and protein digestive enzymatic medicine on the market.

As explained, the study of *Sarcodon aspratus* (Berk.) S. Ito is limited to the property, purification, medicinal action, and feasibility as digestives of protease. Thus, we intended to purify and characterize the protease of *Sarcodon aspratus* (Berk.) S. Ito.

II. Materials and Methods

I. Declared materials

A. Materials

Sarcodon aspratus (Berk.) S. Ito used as a declared material in the experiment was sampled from Kimhae in southern part of Kyungsang province. It was freeze-dried to be used for the blank test material.

B. Reagents and Apparatus

1) Reagents

SDS-PAGE reagents and the reagents for protease activity measurement were those of Sigma Co. DEAE-cellulose(DE52) was from Whatman Co. and others not addressed here were all laboratory reagents.

2) Apparatus

The apparatus used were centrifuge(Sephacryl-S 200, Beckman Co.), mono S column(LKB), chromatography (Pharmacia Co.), and electrophoresis (Bio-rad Co.), etc.

2. Methods

A. Purification of enzymes

1) Dispensing of crude ezyme

Lyophilzed *Sarcodon aspratus* (Berk.) S. Ito was milled(80 mesh), defatted with acetone in 1:5 volume ratio, and dried in the shade. 0.1M Phosphate buffer solution (pH 7.0) was added to 100g of defatted *Sarcodon aspratus* (Berk.) S. Ito . This proceeded to shaking extraction for 2 hours. It was filtered with cheese cloth and the filtrate was centrifuged at 16,000 ×g for 20 minutes. The supernatant was collected to be used as crude-enzyme.

2) Fractionation with $(NH_4)_2SO_4$

$(NH_4)_2SO_4$ was added to the crude-enzyme to make 25% saturated solution. It was centrifuged at 16,000xg for 20 minutes to remove precipitates.The supernatant was added with $(NH_4)_2SO_4$ to make 75% saturated solution. The solution was settled for 2 hours at 4℃ and the centrifugation pross was repeated to collect the precipitates. The precipitates was dissolved in small quantity of the same buffer solution and dialyzed overnight with the same buffer solution in 1:50 volume ratio at 4℃ .

3) DE52 anion exchange column chromatography

Crude-enzyme fractionated with $(NH_4)_2SO_4$ was dissolved in the same buffer solution and injected into DE52 anion exchange column(4×27㎝) balanced with 20mM Tris-HCl buffer(pH 8.0). It was washed as a flow rate of 0.5 ml/min for the first 500 minutes and then fractionated by 5ml with a fraction collector to collect the active portion by gradient elution treatment (0.5ml/min) with 1000ml of the same buffer solution.

4) Sephacryl-S 200 column chromatography

Enzyme dialyzed and concentrated after purification from DE 52 anion exchange column chromatography was injected into Sephacryl-S 200 column(1.5×100㎝) balanced with 0.1M NaCl containing 20mM Tris-HCl buffer(pH 8.0). This was eluted at a vate of 0.1 ml/min to fractionate by 2ml and collect active portions.

5) Mono S column chromatography

Crude-enzyme assay dialyzed and concentrated after purification in Sephacryl-S 200 was dissolved and injected into Mono S column balanced with 10mM citrate-20mM Na_2HPO_4(pH 4.0) buffer. Through gradient elution(0.1ml/min) with 0.5M NaCl containing 10mM citrate-20mM Na_2HPO_4(pH 4.0) buffer, it was fractionated by 0.5ml with fraction collector to collect

the active parts.

B. Measurement of enzyme activity

1) Measurement of protease activity

Enzyme 0.1ml was added to 0.5ml casein solution made soluble with 0.1M phosphate buffer(pH 7.0). It was reacted in 40℃ controlled water bath for 5 minutes, to which 5% trichloroacetic acid(TCA) 0.8ml was added to stop the reaction. The mixture was centrifuged at 20,000xg for 5 minutes.

The supernatant was diluted 2 times and measured the absorbance at 280nm. The difference from the control was presented as an enzyme activity.

C. Measurement of protein concentration

Using bovine serum albumin as a standard protein, protein concentration was measured according to the methods used by Bradford[17] .

D. Measurement of molecular weight with SDS-PAGE

Fractionated amount and molecular weight of protease were analyzed via SDS-PAGE following Laemmli's[18] methods. The concentration of stacking gel and separating gel were 4%, 12%, respectively, and molecular weight marker was prestaining marker (Sigma Co.) in which trypsin inhibitor(M.W.20,100) and α-lactalbumin(M.W.14,200) were mixed. Coomassie brilliant blue R-250 was used to color protein bands and 30% methanol for decoloring.

E. Propterties of enzymes

1) pH Stability

Enzyme was treated to be soluble with 0.1M citrate-0.2M Na_2HPO_4 buffer from pH3.0 to pH6.0, Tris-HCl buffer from pH 7.0 to pH 10.0, carbonate-bicarbonate buffer from pH 10.0 to pH 11.5 It was incubated at 4℃ for 48 hours and analyzed the protease activity.

2) Temperature stability

Enzyme was treated soluble in 0.1M Na_2CO_3-$NaHCO_3$ buffer (pH10.0) and reacted for 30 minutes at each temperature from 30℃ to 90℃. It was chilled in ice water and analyzed its remaining activity to show relative activity.

3) Effect of metal ions and interfering materials

$BaCl_2$, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_2$, $MgSO_4$, $MnSO_4$, $NaCl_2$, KCl, $Pb(CH_3COO)_2$, $ZnCl_2$ and PMSF, EDTA, 2-mercaptoethanol, and SDS was added to enzyme to make 5mM concentration, incubated at 30℃ for 1 hour, and measured the enzyme activity.

III. Results and Discusstion

1. Purification of enzyme

Fig. 1 shows the result of enzyme purification with DE 52 anion exchange column chromatography. The protease of *Sarcodon aspratus* (Berk.) S. Ito was eluted at around 0.2M of NaCl and the activity showed a single peak, which indicated that it had quite much purification effect.

To purify this further, the active parts only was concentrated with amicon and then dialyzed with 20mM Tris-HCl buffer solution(pH 8.0). The results of Sephacryl-S 200 column chromatography are shown in Fig. 2.

Protease activity appeared between frcation No. 50 and No. 65. However, as enzyme protein was not fractionated in a single peak, only the active parts was concentrated with amicon and dialyzed. It was purified further by Mono S column chromatography and the results are in Fig. 3.

The protease activity of *Sarcodon aspratus* (Berk.) S. Ito showed a single peak between fraction No. 14 and No. 17.

All the elution was collected and dialyzed for electrophoresis to determine if it was purified completely. The result, as in Fig. 4, showed a single band, which meant it was almost completely purified. When plotting this to the molecular weight and the Rf value of standard protein, molecular weight as shown in Fig. 5 was estimated 29,300.

This is similar to 30,100 reported by Lee[19]and 29,000 by Eun et al[20]. as the molecular weight of protease Also, in Fig. 4, the protease of purified *Sarcodon aspratus* (Berk.) S. Ito appeared as a single band, this enzyme is thought to be monomer.

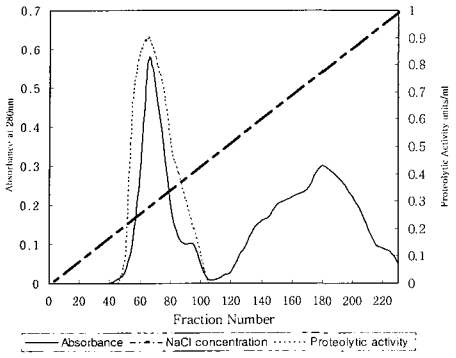

Fig. 1. DE52 anion exchange column chromatography from *Sarcondon aspratus.*

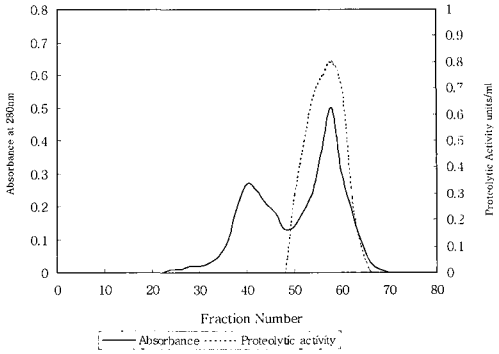

Fig. 2. Sephacryl-S 200 column chromatography from *Sarcodon aspratus.*

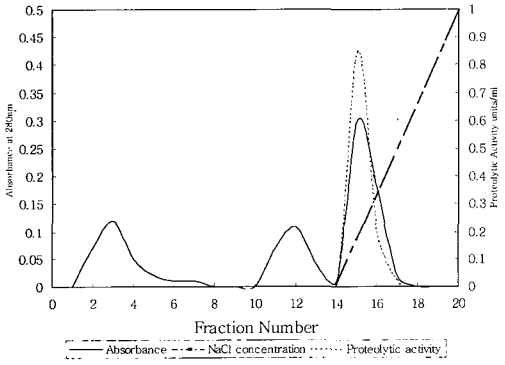

Fig. 3. Mono S column chromatography from *Sarcodon asprattus.*

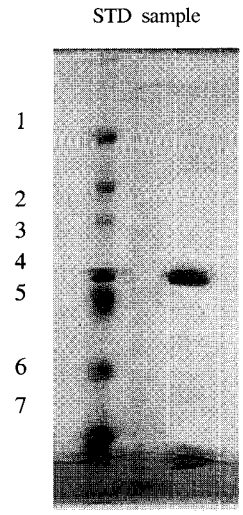

Fig. 4. SDS-PAGE assay of the purified enzyme. Molecular weight of the protein was calculated by comparison with seventh standard proteins(STD):1-Bovine serum albumin (66,000), 2-Ovoalbumin (45,000), 3-GAP-dehydrogenase (36,000), 4-Carbonic anhydrase (29,000), 5-Trypsinogen (24,000), 6-Trypsin inhibitor (20,100), 7-α -lactalbumin (14,200)

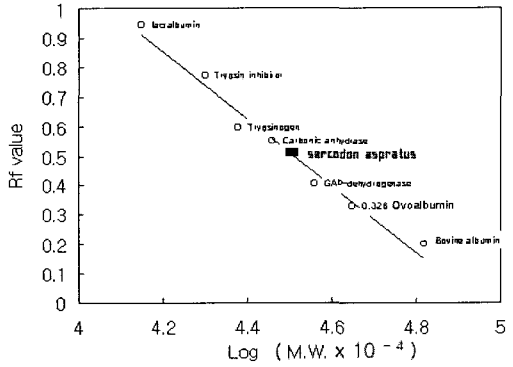

Fig. 5. Molecular weight determination of the enzyme by SDS-PAGE

The overall results of protease purification of *Sarcodon aspratus* (Berk.) S. Ito is listed in Table 1. The specific activity of crude-enzyme was 4.9 U/mg protein but 55.2 U/mg protein in Mono S column chromatography, 11.26 times more than crude ezyme, with yield 49.5%. This result shows that the enzyme are as much as active according to the lower times for the purification to the enzymes, which results from lots of enzymes contained in the dried mushroom.

Table. 1. Purification of the proteolytic enzyme from *Sarcodon aspratus*

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yeild (%)	Purity (fold)
Crude enzyme	841	4125	4.9	100	1
75% $(NH_4)_2SO_4$	328	3730	11.4	90.4	2.3
DE52 anion	167	3277	19.6	79.4	4.0
Sephacryl S-200	92	2863	31.1	69.4	6.3
Mono S	37	2041	55.2	49.5	11.3

2. The property of the enzyme

1) pH Stability

When the enzyme was preserved at 4℃ for 48 hours, pH stability of the enzyme was as shown in Fig. 6, most stable at pH 7.5, highly active in the range of pH 5.5~pH 10.5, and rapidly decreasing pH 10.5 and became 0.089 (U/ml) at pH 11.5. This result is similar to the result of Lee's[19] result who separated and purified protease from Sarcodon aspratus. According to Lee[19] the protease activity was most stable around pH 7.0 and stable in the wide range of pH from 3.0 to 10.8, but decreasing rapidly over pH 10.8.

2) Temperature stability

To analyze the temperature stability of the enzyme, casein as a substrate was preserved at each temperature for 30 minutes and analyzed the enzyme activity. The results are in Fig. 7. Protease maintained comparatively the initial activity till 50℃ but completely lost the activity over 50℃. This is higher than Lee's[19] result that the activity remained 26% at 60℃, and similar to Lee[16] who analyzed the temperature stability with Crude-enzyme of Sarcodon aspratus.

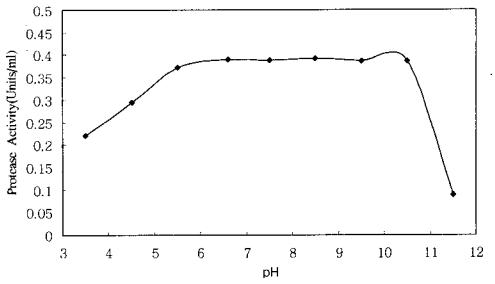

Fig. 6. pH stability of the purified enzyme from *Sarcondon aspratus*

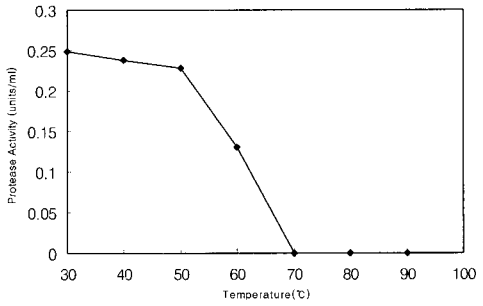

Fig. 7. Effect of temperature on the protease activity of the purified enzyme from *Sarcodon aspratus*

3) Metal ions and interfering materials.

Various metal ions and interfering materials were added to make 5mM concentration and analyze the enzyme activity. The result is in Fig. 9.

Protein degradability of the enzyme was not that much interfered by metal ions. Among the interfering materials tested to protease from *Sarcodon aspratus*, SDS turned out to be most interfering. 5mM used in

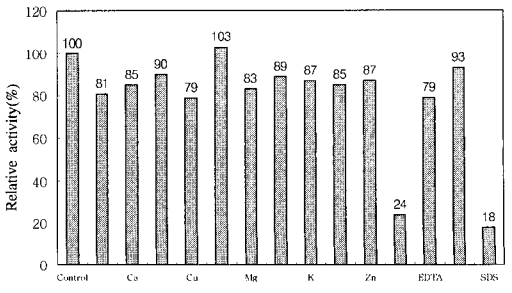

Fig. 8. Effect of metal ions and inhibitors on the protease activity from *Sarcodon aspratus*

the study was about 0.14%, it was not equal to Eun et al[20] reported 0.2% to be stable in SDS -PAGE. While, the protein degrading activity of enzyme toward interfering materials was highly interfered by PMSF, which means that the enzyme could be serine protease.

Ⅳ. Conclusion

The protease from *Sarcodon aspratus*(Berk.) S. Ito was extracted and purified through salt out with 70%$(NH_4)_2SO_4$, DE52 anion exchange column chromatography, and Sepharyl-S200 column and Mono S column chromatography. The specific activity was 55.2u/mg protein, 11.26 times more than that of crude enzyme, with yield of 49.5%.

The electrophoresis of the purified enzyme showed a single band and estimated the molecular weight to be 29,300.

The pH stability, when preserved at 4℃ for 48 hours, was most stable at pH 8.5 and kept high activity in the range of pH 5.5～10.5.

As for the temperature stability when preserved for 30 minutes at each temperature, the protein degradability kept comparatively the initial activity, 53% of the activity at 60℃, lost it rapidly over that temperature, and lost completely at 70℃.

Metal ions didn't show interfering effect that much but SDS and PMSF did, which indicates that the enzyme could be serine protease.

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