Purification and Some Properties of Protease Produced by *Staphylococcus hyicus* subsp. *hyicus* strain No. 111

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ABSTRACT. The purification of protease, produced by *Staphylococcus hyicus* subsp. *hyicus* strain No. 111, was performed by successive precipitations with ammonium sulfate and cold acetone, chromatography on DEAE-cellulose and gel filtration on Sephadex G-75. By the procedure, the protease was purified 474.1-fold, and its recovery rate was 34.9%. The purity of the preparation was demonstrated by polyacrylamide gel electrophoresis, the immunodiffusion test and immunoelectrophoresis. The protease was most active at a neutral value of pH. The protease activity was inhibited by the presence of Cu, Zn, Ag, Fe, or Hg ions, but was slightly stimulated by Ca ion. No protease was stimulated by the reducing agents such as cysteine, 2-mercaptoethanol and sodium thioglycolate, and it was strongly inhibited by the addition of EDTA. The molecular weight of the purified protease was estimated to be 32,000 by SDS-polyacrylamide gel electrophoresis.—**KEY WORDS:** protease, *S. hyicus* subsp. *hyicus*, swine exudative epidermatitis.

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Staphylococcus hyicus subsp. *hyicus* is frequently isolated from pigs affected with exudative epidermatitis [5, 10, 15]. In addition, exudative epidermatitis can be reproduced by inoculating *S. hyicus* subsp. *hyicus* into young pigs [1, 9, 12]. Therefore, *S. hyicus* subsp. *hyicus* is considered to be an etiologic agent of swine exudative epidermatitis.

Devriese *et al.* [5, 6] reported that almost all strains of *S. hyicus* subsp. *hyicus* produce various proteolytic enzymes, which hydrolyze some protein substances such as skim milk, gelatin and fibrin *in vitro*. But the purification and properties of these proteases still have to be investigated. In the previous experiment [18], the authors found that strain No. 111 of *S. hyicus* subsp. *hyicus*, isolated from a pig suffering from exudative epidermatitis, showed strong casein hydrolytic ability. Then the authors attempted to purify the protease produced by strain No. 111 and to examine some of its properties.

MATERIALS AND METHODS

Microorganisms and cultivation: *S. hyicus* subsp. *hyicus* strain No. 111, which had been isolated from a pig suffering from exudative epidermatitis, was used throughout the experiment. The strain produced a remarkable amount of protease *in vitro* in comparison with other strains. The organisms were inoculated into 200 ml of Heart infusion broth (Difco) in a Sakaguchi's flask of 500 ml capacity and cultivated on a shaker at 37°C for 18 hours.

Assay of proteolytic activity: Proteolytic activity was determined by the method of Kunitz [11]. Heat-denatured casein (Wako) was used as a substrate. To each 0.5 ml of the diluted enzyme solution in 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM CaCl₂, 2 ml of 1% casein in 0.01 M phosphate buffered saline, pH 7.2, were added. The resulting mixtures were incubated in a water bath at

37°C for 30 minutes, and then the reaction was stopped by the addition of 3 ml of 10% perchloric acid. The resulting precipitate was removed by filtration through Toyo No. 6 filter paper. The optical density of the filtrate at 280 nm was determined by a Hitachi spectrophotometer. The filtrate obtained from the mixture, which was acidified soon after mixing, was used as a blank. The potency of the enzyme was expressed as the optical density at 280 nm; that is, one optical density was defined as one unit.

Determination of protein: The protein contents of samples taken during the purification procedure were determined by the modified Folin method [13]. Bovine serum albumin (Wako) was used as a standard. In some cases protein concentration was represented with an optical density at 280 nm measured by a Hitachi spectrophotometer.

Polyacrylamide gel and SDS-polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis was performed in 7% gel with Tris-glycine buffer, pH 8.3, according to the method of Davis [4]. The sample was applied to the gel column and run at 3 mA per column for 4 hours at room temperature. SDS-polyacrylamide gel electrophoresis was performed by the method of Shapiro *et al.* [14] and Dnker and Rueckert [8]. The sample was heated in a boiling water bath for 2 minutes in the presence of 1% 2-mercaptoethanol, 1% SDS and 20% glycerin. After that, electrophoresis was carried out with a constant current of 8 mA at room temperature. The gels were stained with Coomassie Brilliant Blue R-250.

Molecular weight determination: The molecular weight determination of purified protease was performed by SDS-polyacrylamide gel electrophoresis as mentioned above. An electrophoresis calibration kit for low molecular weight (Pharmacia) was used as the reference proteins. The molecular weight of the protease was determined from a linear slope of the logarithm of the molecular

weight of these standard proteins against Rf values.

Preparation of antiserum: The culture supernatant was concentrated to 1/10 volume by the precipitation with ammonium sulfate of 80% saturation. After being dialyzed, 1 ml of the concentrated solution was emulsified in an equal amount of Freund's incomplete adjuvant and inoculated intramuscularly into a rabbit three times at intervals of two weeks. Serum was taken from the rabbit three weeks after the last inoculation.

Immunodiffusion test and immunoelectrophoresis: The immunodiffusion test and immunoelectrophoresis were carried out by the procedures described previously [16].

RESULTS

Purification of protease: Solid ammonium sulfate was added to the culture supernatant until a concentration of 75% saturation was reached. After stirring, the resulting precipitate was collected by centrifugation and dissolved in 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM CaCl₂. Then, cold acetone was added slowly with stirring to the dissolved solution in the ratio of 1.4:1. The mixture was immediately centrifuged, and the precipitate obtained was dissolved in the same buffer. After being dialyzed against 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM CaCl₂, the solution was applied to a column (1.6×40 cm) of DEAE-cellulose (Whatman, DE-52), which had been previously equilibrated with the same buffer. The column was washed thoroughly with the starting buffer and then was subjected to a linear gradient elution. In the elution, 200 ml of the starting buffer and 200 ml of buffer containing 0.6 M KCl was applied. As shown in Fig. 1, the proteolytic activity was eluted in the first peak.

The active fractions were pooled and concentrated with industrial carboxymethyl cellulose. The concentrated solution was applied to a Sephadex G-75 column (2.6×90

Fig. 1. Chromatography on a DEAE-cellulose column of the preparation obtained after acetone precipitation.

cm) and eluted with 0.01 M Tris-HCl buffer, pH 7.5, containing 1 mM CaCl_2 . As shown in Fig. 2, two protein peaks were detected by absorption at 280 nm. The proteolytic activity was seen in the first peak. The active fractions were pooled and subjected again to gel filtration using a Sephadex G-75 column under the same conditions as mentioned above.

The recovery rate and degree of purity of protease in each step of purification are summarized in Table 1. The final yield of enzyme activity was 34.9%, with a purification degree of 474.1-fold.

Purity of the purified protease was examined by polyacrylamide gel and SDS-polyacrylamide gel electrophoresis. As shown in Fig.

3, the protease formed only one band in both gels, respectively. In addition, only one precipitating line was observed when the purified protease was examined by the immunodiffusion test (Fig. 4) and by immunoelectrophoresis using the antiserum.

Some properties of protease: The ultraviolet absorption at various wavelengths of the purified protease was determined by a Hitachi spectrophotometer. The protease showed a similar curve, which was the typical absorption curve of protein.

Optimum pH was determined by using casein dissolved in buffers at various pH values. As a result, the optimum pH of protease was found at pH 7.4.

The protease solutions were allowed to

Fig. 2. Gel filtration on a Sephadex G-75 column of protease obtained by chromatography on a DEAE-cellulose column.

Table 1. Purification of protease produced by *S. hyicus* subsp. *hyicus* strain No. 111

	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Specific activity	Purification (fold)	Recovery (%)
Culture supernatant	2,700	6.60	4.3	0.6	1	100
Ammonium sulfate	220	1.05	37.5	35.7	59.5	71.0
Cold acetone	50	2.13	146.5	68.7	114.5	63.0
DEAE-cellulose	36	0.91	168.0	184.6	307.6	52.0
Sephadex G-75	55	0.41	103.0	251.2	418.3	48.7
Re-Sephadex G-75	46	0.28	88.2	284.5	474.1	34.9

stand at various temperatures for 10 minutes, and then they were cooled rapidly for the examination of remaining activity. When incubated at 50°C, protease showed no significant loss, but after incubation at 60°C, it lost about 95% of the original activity.

The effects of various agents on the proteolytic activity are shown in Tables 2 and 3. The protease was inactivated in the presence of Cu, Zn, Ag, Fe and Hg ions at a concentration of 1 mM. The enzyme was slightly

stimulated by the presence of Ca ion. On the other hand, no protease was stimulated by reducing agents such as cysteine, 2-mercaptoethanol and sodium thioglycolate. In particular, it was inactivated by the presence of EDTA.

The molecular weight of the purified protease was determined by SDS-polyacrylamide gel electrophoresis. A plot of the logarithm of the molecular weights of standard proteins against mobility is shown in Fig. 5. By

Fig. 3. Polyacrylamide gel (1) and SDS-polyacrylamide gel (2) electrophoresis of purified protease.

Fig. 4. Immunodiffusion reaction between anti-crude protease serum and samples at different purification steps.

- A; Anti-crude protease serum
 1; Ammonium sulfate precipitation
 2; Acetone fractionation
 3; DEAE-cellulose chromatography
 4; First gel filtration on Sephadex G-75
 5; Second gel filtration on Sephadex G-75

Table 2. Effect of divalent cations on the activity of protease

Reagent (1 mM)	Relative activity (%)
None	100
BaCl ₂	101
CuSO ₄	6
ZnSO ₄	9
(NH ₄) ₂ SO ₄	115
MgSO ₄	99
AgNO ₃	5
FeSO ₄	8
MnCl ₂	97
CaCl ₂	110
HgCl ₂	6

Table 3. Effect of some reagents on the activity of protease

Reagent	Relative activity
None	100%
Cysteine (1 mM)	61
Cysteine (10 mM)	10
2-Mercaptoethanol (1 mM)	89
2-Mercaptoethanol (10 mM)	93
Sodium thioglycolate (1 mM)	95
Sodium thioglycolate (10 mM)	61
Sodium citrate (1 mM)	30
Sodium citrate (10 mM)	11
EDTA (1 mM)	9
EDTA (10 mM)	10

interpolation, the molecular weight of the protease was estimated to be 32,000.

DISCUSSION

Some extracellular proteases, produced by *Staphylococcus aureus*, have been purified and characterized. Drapeau *et al.* [7] purified

a protease from the culture supernatant of *S. aureus* strain V8. The protease was inhibited by diisopropyl fluorophosphate, but not by EDTA. Arvidson *et al.* [2, 3] purified three proteases (Protease 1, 2, and 3) from *S. aureus* strain V8 and characterized. The activity of protease 1 was not significantly affected by divalent cations, sulfhydryl rea-

Fig. 5. Determination of the molecular weight of protease by SDS-polyacrylamide gel electrophoresis.

L; Alpha-lactalbumin (14,400)
 T; Trypsin inhibitor (20,100)
 C; Carbonic anhydrase (30,000)
 O; Ovalbumin (43,000)
 A; Albumin (67,000)
 P; Phosphorylase b (94,000)

gents, EDTA, or diisopropyl fluorophosphate. On the contrary, protease 2 was active only in the presence of reducing agents, and it was inactivated by heavy metals. Protease 3 was an EDTA-sensitive enzyme. The authors [17] purified previously two proteases produced by *S. aureus* strain CH-91, which was isolated from a chicken suffering from dermatitis. The activity of the proteases was strongly stimulated by reducing agents such as potassium cyanide and thioglycolate.

In the present experiment, the authors purified a protease of *S. hyicus* subsp. *hyicus* by precipitations with ammonium sulfate and cold acetone, chromatography on DEAE-cellulose, and gel filtration on Sephadex G-75. The purification procedures, except gel filtration, were similar to those of Drapeau [7]. The protease thus obtained was inactivated by

EDTA and was not stimulated by the reducing agents used. Calcium was necessary for the stability of the protease. From the results, it seems that the protease produced by *S. hyicus* subsp. *hyicus* strain No. 111 may belong to protease 3 of *S. aureus*, which was classified by Arvidson [3].

Almost all strains of *S. hyicus* subsp. *hyicus* produce some proteases which hydrolyze skim milk (casein). Moreover, some strains have strong protease producing ability. Though the results were not described in this paper, the antiserum against the protease of strain No. 111 did not react to the proteases of any of the strains when examined by the immunodiffusion test. Therefore, it seems that the protease of strain No. 111, which was purified in this investigation, may not be a typical protease of *S. hyicus* subsp. *hyicus*. On the other hand, almost all the strains of *S. hyicus* subsp. *hyicus* have strong gelatin and fibrin hydrolytic ability. Therefore, it is necessary to purify these proteolytic enzymes in order to clear the virulence factors of *S. hyicus* subsp. *hyicus*.

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REFERENCES

1. Amtsberg, G., Bollwahn, W., Hazem, S., Jordan, B., und Schmidt, U. 1973. Bakteriologische, serologische und tierexperimentelle Untersuchungen zur ätiologischen Bedeutung von *Staphylococcus hyicus* beim nasendem Ekzem des Schweines. *Dtsch. Tierarztl. Wochenschr.* 80: 493-499.
2. Arvidson, S., Holme, T., and Lindholm, B. 1973. Studies on extracellular proteolytic enzymes from *Staphylococcus aureus*. I. Purification and characterization of one neutral and one alkaline protease. *Biochem. Biophys. Acta.* 302: 135-148.
3. Arvidson, S. 1973. Studies on extracellular proteolytic enzymes from *Staphylococcus aureus*. II. Isolation and characterization of an EDTA-sensitive protease. *Biochem. Biophys. Acta.* 302: 149-157.

4. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 12: 404-427.
5. Devriese, L. A. 1977. Isolation and identification of *Staphylococcus hyicus*. *Am. J. Vet. Res.* 38: 787-792.
6. Deveriese, L. A., Hájek, V., Oeding, P., Meyer, S. A., and Schleifer, K. H. 1978. *Staphylococcus hyicus* (Sompolinsky 1953) comb. nov. and *Staphylococcus hyicus* subsp. *chromogenes* nov. *Inter. J. Sys. Bacteriol.* 28: 482-490.
7. Drapeau, G. R., Boily, Y., and Houmard, J. 1972. Purification and properties of an extracellular protease of *Staphylococcus aureus*. *J. Bio. Chem.* 247: 6720-6726.
8. Dunker, A. K., and Rueckert, R. R. 1969. Observations on molecular weight determinations on polyacrylamide gel. *J. Biol. Chem.* 244: 5074-5080.
9. Jones, L. D. 1956. Exudative epidermitis of pigs. *Am. J. Vet. Res.* 17: 179-193.
10. Kawano, J., Shimizu, A., and Kimura, S. 1983. Bacteriophage typing of *Staphylococcus hyicus* subsp. *hyicus* isolated from pigs. *Am. J. Vet. Res.* 44: 1476-1479.
11. Kunitz, M. 1947. Crystalline soybean trypsin inhibitor. II. General properties. *J. Gen. Physiol.* 30: 291-310.
12. L'Ecuyer, C. 1966. Exudative epidermitis in pigs. *Can. J. Comp. Med.* 30: 9-16.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
14. Shapiro, A. L., Vinuela, E., and Maizel, J. V. Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* 28: 815-820.
15. Sompolinsky, D. 1953. De l'impetigo contagiosa suis et du *Micrococcus hyicus* n. sp. *Schweiz. Arch. Tierheilkd.* 95: 302-309.
16. Takeuchi, S., and Suto, T. 1973. Purification and serological properties of staphylococcal nuclease. *Natl. Inst. Anim. Health. Q (Jpn).* 13: 196-202.
17. Takeuchi, S., and Suto, T. 1974. Purification and properties protease produced by *Staphylococcus aureus* strain CH-91 isolated from diseased chickens. *Natl. Inst. Anim. Health. Q (Jpn).* 14: 121-128.
18. Takeuchi, S., Kobayashi, Y., Morozumi, T., and Niibori, S. Isolation and some properties of *Staphylococcus hyicus* subsp. *hyicus* from pigs, chickens and cows. *Jpn. J. Vet. Sci.* (submitted).

要 約

Staphylococcus hyicus subsp. *hyicus* No. 111 株のプロテアーゼの精製と性状: 竹内正太郎・小林良則・両角徹雄 (家畜衛生試験場北陸支場)——*Staphylococcus hyicus* subsp. *hyicus* No. 111 株のプロテアーゼを硫酸塩析, 冷アセトン没澱, DEAE セルロース・クロマトグラフィーおよびセファデックス G-75・ゲルろ過によって精製した。この方法によって, プロテアーゼは 474.1 倍に精製され, その回収率は 34.9% であった。この精製標品の純度はポリアクリルアミドゲル電気泳動, 寒天ゲル内沈降反応および免疫電気泳動によって証明された。このプロテアーゼは中性の pH で最も活性を示し, その活性は Cu, Ag, Fe, Hg イオンで阻止されたが, Ca イオンでは, わずかに促進された。また, このプロテアーゼはシステイン, 2メルカプトエタノールおよびチオグリコール酸ナトリウムなどの還元剤によって活性化されず, EDTA によって阻止された。精製プロテアーゼの分子量は SDS-ポリアクリルアミドゲル電気泳動によって 32,000 と測定された。