Biochimica et Biophysica Acta, 350 (1974) 38–47

BBA 67204

STUDIES ON PROTEINASES FROM *FICUS CARICA* VAR. HŌRAISHI

V. PURIFICATION AND PROPERTIES OF A SUGAR-CONTAINING PROTEINASE (FICIN S)

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(Received December 27th, 1973)

SUMMARY

A sugar-containing proteinase (Ficin S, EC 3.4.4.12) from *Ficus carica* var. Hōraishi was purified by CM-cellulose and CM-Sephadex C-50 and crystallized. The purified Ficin S was electrophoretically homogeneous.

The sugar content of Ficin S was determined to be 4.8% with the phenol–H_2SO_4 method. It is considered that the sugar is tightly bound to enzyme protein, because the mobility of the protein band coincided with that of the sugar one in disc electrophoresis and the sugar content was constant before and after isoelectric focusing of the enzyme (p*I*, 9.1).

A molecular weight of $2.6 \cdot 10^4$ for Ficin S was obtained by sodium dodecylsulfate–polyacrylamide gel electrophoresis. The enzyme was most active at pH 8.0 and 60 °C, and stable over a pH range 2.0–8.0 at 4 °C for 20 h and below 60 °C for 30 min. The enzyme was activated by cysteine and mercaptoethanol, but inhibited particularly by $HgCl_2$ and *p*-chloromercuribenzoate. These properties of Ficin S were discussed in comparison with the other enzymes i.e. Ficin A, B, C and D from *Ficus carica* var. Hōraishi.

These results suggest that Ficin S differs only in isoelectric point and sugar content from Ficin A, B, C and D.

INTRODUCTION

Sgarvieri [1] has reported that the latex of many varieties of *Ficus carica* and *Ficus glabrata* contains a number of sulfhydryl proteinases. It appears that the various proteinases from a single variety of tree are virtually indistinguishable in terms of kinetics and specificity, have a similar molecular weight, and show considerable similarity in primary structure, as judged by peptide mapping [2, 3]. However, the enzymes exhibit clear differences in amino acid composition, peptide maps, and chromatographic and electrophoretic properties [3].

In previous papers [4–6], the authors have also reported that the latex from

Abbreviation: Tos-Arg-OMe, α-*N*-tosyl-L-arginine methylester.

Ficus carica var. Hōraishi natively contains four proteinases (Ficin A, B, C and D) which are distinguishable in isoelectric point, chromatographic behavior, thermal stability, amino acid composition and primary structures as examined by tryptic fingerprints.

Sugar-containing proteinase (Ficin S) has also been found in the latex of *Ficus carica* var. Hōraishi. The authors purified the Ficin S from the latex in order to clarify the multiplicity of the proteinases. In this paper, some properties of the purified enzyme are described in comparision with those of Ficin A, B, C and D.

MATERIALS AND METHODS

Enzyme

Latex from the green fruits of *Ficus carica* var. Hōraishi was collected between late August and early October at Gifu city (Japan). The gum was removed from the latex by freezing and thawing followed by centrifugation at 10 000 rev./min for 20 min and the straw-colored aqueous solution (crude enzyme) was frozen and stored at −20 °C. Ficin A, B, C and D were purified from the crude enzyme as described by Sugiura and Sasaki [4].

Reagents

CM-cellulose (0.72 mequiv/g) was the product of Brown. CM-Sephadex C-50 and Sephadex G-50 (fine) were products obtained from Pharmacia Fine Chemicals A.B. α-*N*-tosyl-L-arginine methylester (Tos-Arg-OMe) was obtained from Nakarai Chemicals Ltd. Casein (Hammersten quality) was from E. Merck. Bovine serum albumin and cytochrome *c* (Sigma), egg albumin (Nutritional Biochemicals Corp.), pepsin (Miles), trypsinogen (Miles-Seravac) and myoglobin (Calbiochem.) were used as the standards for the determination of the molecular weight of Ficin S. $Na_2S_4O_6$ was prepared by the method of Gilman [7] and was stored at 4 °C in a desiccator. The other chemicals used were of special or reagent grade.

Assay of enzyme activity

Proteolytic activity was measured by the method of Kunitz [8] in the presence of 2% casein and 0.025 M cysteine at pH 7.0 and 37 °C. Specific activity was expressed as the initial change in absorbance at 280 nm per min per mg of enzyme protein.

The activity toward Tos-Arg-OMe was determined by measuring the increase in absorbance at 247 nm according to Hummer [9]. Before measuring the activity, the enzyme was activated by 0.01 M cysteine at pH 7.0 and 37 °C for 10 min. Protein concentration was determined by measuring the absorbance with a Beckman DU-2 spectrophotometer. $\varepsilon_1^{1\%}{}_{cm}$ at 280 nm for Ficin S was assumed to be 20.

Measurement of hydrolysis ratio of casein

To a 2% casein solution (pH 7.0), 3.3 units/g casein of the enzyme was added and the hydrolysis was carried out in the presence of 0.025 M mercaptoethanol at 37°C for 48 h. The extent of the hydrolysis of casein was measured by the formol titration method [10]. The value of 100% was attained in the case that casein was hydrolyzed by 6 M HCl at 110 °C for 48 h.

Isoelectric focusing and disc electrophoresis

Isoelectric focusing was carried out as described by Vesterberg and Svensson [11] and disc electrophoresis was performed by the procedure of Reisfeld [12] and Nagai [13]. Protein was stained with Amidoschwartz 10B and sugar was detected with fuchsin–sulfite stain after periodic acid oxidation [14].

Determination of molecular weight and estimation of sugar

The determination of molecular weight was carried out with sodium dodecyl-sulfate polyacrylamide gel electrophoresis [15]. Estimation of sugar was carried out with the phenol–H_2SO_4 method as described by Dubois [16] using glucose as the standard.

RESULTS

Purification and crystallization of Ficin S

Crude enzyme was separated into 7 fractions with a CM-cellulose column as shown in Fig. 1. In the previous paper [4], it was reported that Ficin A, B, C and D

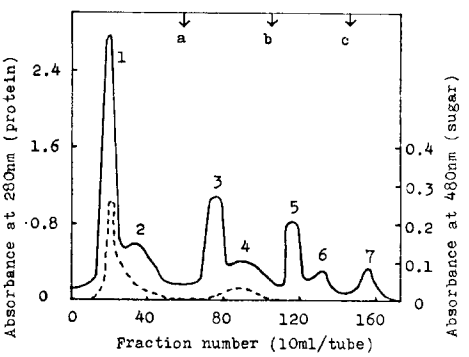

Fig. 1. Chromatography of crude enzyme from *Ficus carica* var. Hōraishi. 1, Fraction 1; 2, Fraction 2; 3, Fraction 3; 4, Fraction 4; 5, Fraction 5; 6, Fraction 6; 7, Fraction 7. 20 ml of CM-cellulose adsorbed with crude enzyme (350 mg) was placed on top of a CM-cellulose column (1.65 cm × 50 cm) equilibrated with 0.01 M sodium phosphate buffer containing 0.08 M NaCl and 1 mM $Na_2S_4O_6$ (pH 7.0). Following elution with the starting buffer, stepwise elution was made with NaCl as indicated by arrows: a, 0.13 M NaCl; b, 0.22 M NaCl; c, 0.4 M NaCl, all in 1 mM $Na_2S_4O_6$ and 0.01 M sodium phosphate buffer (pH 7.0). ———, protein; — — —, sugar.

were obtained from the Fractions 1, 3, 5 and 7 in Fig. 1, respectively. Sugar was contained in Fractions 1 and 4, respectively. Since the sugar content of Fraction 1 was higher than that of Fraction 4, Fraction 1 was selected for the purification of Ficin S. Fraction 1 was subjected to CM-cellulose column chromatography and the result is shown in Fig. 2a. The sugar-containing peak in Fig. 2a was two times rechromatographed on column (2 cm × 50 cm) of CM-cellulose equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 1 mM $Na_2S_4O_6$, with a linear gradient system of NaCl (0–0.1 M). The enzyme showed one peak in the last chromatography and the specific activity was almost constant across the peak, but impurities were detected with disc electrophoresis. The enzyme was further fractionated with a CM-Sephadex

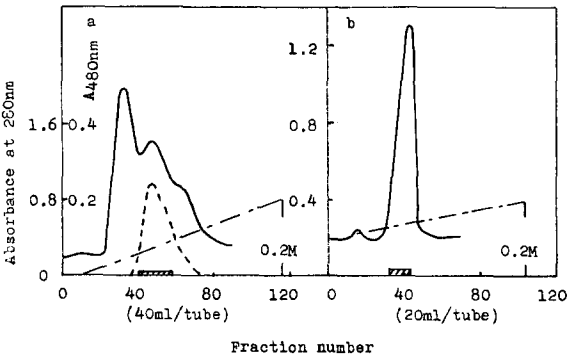

Fig. 2. Purification of Ficin S by column chromatography with CM-cellulose (a) and CM-Sephadex C-50 (b). a. Fraction 1 (1250 mg) in Fig. 1 was adsorbed onto 25 g of CM-cellulose equilibrated with 0.01 M citric acid–0.02 M Na_2HPO_4 buffer (pH 4.5) and placed on the top of a 3.3 cm × 50 cm column equilibrated 0.01 M sodium phosphate buffer (pH 7.0) containing 1 mM $Na_2S_4O_6$. The elution was carried out with a linear gradient system of NaCl (0–0.2 M). The fractions indicated by the cross-hatched bars were used for further purification. b. Twice rechromatographed enzyme was adsorbed onto the CM-Sephadex C-50 column (1.65 cm × 50 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0) containing 1 mM $Na_2S_4O_6$ and 0.01 M NaCl. The elution was carried out with a linear gradient system of NaCl (0.1–0.2 M). The fractions indicated by the cross-hatched bars were gathered for crystallization. ———, absorbance at 280 nm; – – –, absorbance at 480 nm; — — —, concentration of NaCl with linear gradient elution.

C-50 column. The chromatogram is presented in Fig. 2b and impurities were separated from the peak.

The purified enzyme was collected and precipitated by the addition of $(NH_4)_2SO_4$ until the solution was saturated. The precipitate was gathered by centrifugation and dissolved in the minimum amount of 1 mM $Na_2S_4O_6$ and dialyzed against the same solution at 4 °C. Crystals formed in a few days and were gathered and stored at 4 °C. A microphotograph of the crystals is given in Fig. 3.

The results of the purification procedures are summarized in Table I. The enzyme was obtained in a yield of 3.2%. The specific activity was constant throughout the purification, but the sugar content of Ficin S increased up to 4.8%.

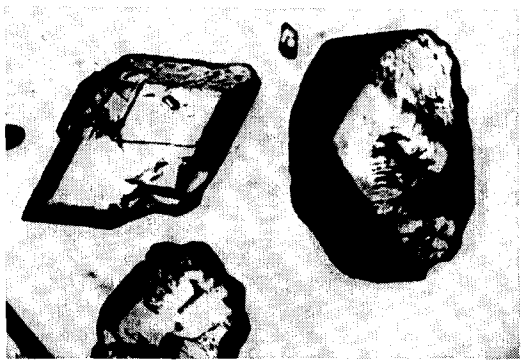

Fig. 3. Crystals of Ficin S from *Ficus carica* var. Hōraishi. Magnification, ×27.

TABLE I

PURIFICATION OF FICIN S FROM *FICUS CARICA* VAR. HŌRAISHI

Step of purification	Activity (units)	Protein (mg)	Spec. act. (units/mg)	Sugar content (%)	Recovery of activity (%)
Fraction 1	2170	1250	1.7	1.8	100
CM-cellulose(1)	930	500	2.0	3.0	43
CM-cellulose(2)	370	210	1.8	4.8	17
CM-cellulose(3)	207	120	1.8	4.8	9.5
CM-Sephadex C-50	77	43	1.8	4.8	3.5
Crystallization	70	39	1.8	4.8	3.2

Homogeneity

The homogeneity of purified Ficin S was examined with disc electrophoresis. An homogeneous single band for protein or sugar is shown in Fig. 4, where the protein band coincided with that of the sugar. As shown in Fig. 4, the mobility of Ficin S in the electrophoresis was different from that of Ficin A, B, C and D. Sugars were not detected in Ficin A, B, C and D with periodate–Schiff reagent [14] following disc electrophoresis and also with the phenol–H_2SO_4 method [16].

Isoelectric point and molecular weight

The isoelectric point of Ficin S was determined by isoelectric focusing using a

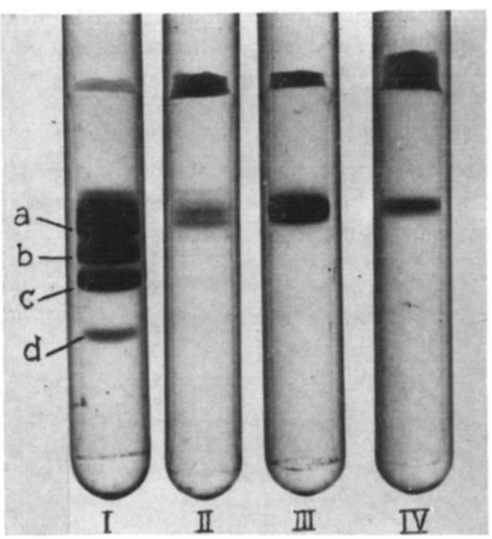

Fig. 4. Disc electrophoretic pattern of crude enzyme and purified Ficin S. About 150 μg of crude enzyme (I, II) and 40 μg of purified Ficin S (III, IV) were subjected to electrophoresis at pH 4.0. A current of 5 mA/tube was supplied for 90 min at 4 °C. Gels I and III were stained with amidoschwarz 10B to visualize the protein and gels II and IV were treated with periodic acid followed by fuchsin-bisulfite to demonstrate sugar. Bands a, b, c and d in gel I indicate the mobility of Ficin A, B, C and D, respectively [4].

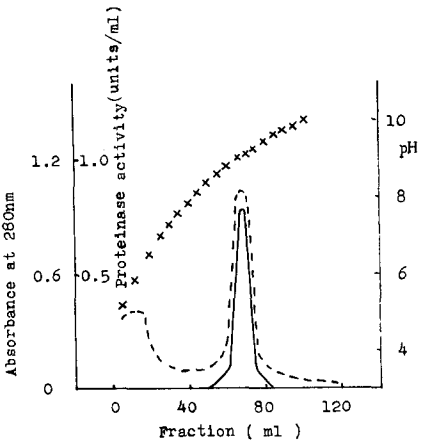

Fig. 5. Isoelectric focusing of Ficin S from *Ficus carica* var. Hōraishi 15 mg of crystalline Ficin S was used. A carrier ampholyte was selected to give a pH range from 7 to 10 and used at a 1% concentration. Electrofocusing was performed for 48 h with a potential of 700 V at 4 °C. The column volume was 110 ml. – – –, protein; ———, proteinase activity; × × ×, pH.

carrier ampholyte (pH 7–10) and the result is shown in Fig. 5. The proteolytic activity appeared in a single peak and the isoelectric point was estimated to be pH 9.1.
From this data, the homogeneity of the purified Ficin S was also proved. For the determination of sugar content in Ficin S after isoelectric focusing, the active fraction was applied onto a column (1.65 cm × 90 cm) of Sephadex G-50 equilibrated with 0.1 M NaCl and 1 mM $Na_2S_4O_6$ to remove carrier ampholyte and sucrose. The sugar content of gel-filtrated Ficin S was determined to be 4.8% and this value was the same as that of purified Ficin S in Table I.

The molecular weight of Ficin S was determined using the method of Dunker [15] with sodium dodecylsulfate–polyacrylamide gel electrophoresis. From the results, as shown in Fig. 6, the value of the molecular weight was calculated to be $2.6 \cdot 10^4$.

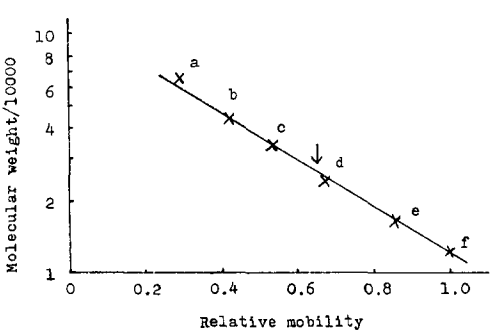

Fig. 6. Determination of the molecular weight of Ficin S using 10% polyacrylamide gel containing 0.1% sodium dodecylsulfate. 20 μg of standard proteins and Ficin S treated with sodium dodecylsulfate was used and the electrophoresis was performed at pH 7.0, 4 °C and 8 mA/tube for 5 h. The mobility was expressed taking that of cytochrome *c* as 1.0. a, bovine serum albumin (mol. wt 69 000); b, egg albumin (mol. wt 43 000); c, pepsin (mol. wt 35 500); d, trypsinogen (mol. wt 24 000); e, myoglobin (mol. wt 16 800); f, cytochrome *c* (mol. wt 12 800). The arrow indicates the mobility of Ficin S.

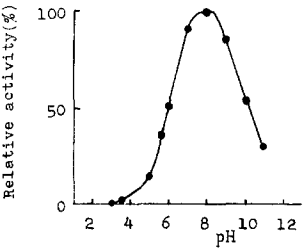

Fig. 7. Effect of pH on activity of Ficin S. Reaction mixture of 4 ml of 1% casein buffered with Britton–Robbinson buffer, 1 ml of 0.15 M cysteine and 1 ml of enzyme solution (0.03 unit) was incubated at various pH for 30 min at 37 °C.

Optimum pH, temperature and stability

The maximum activity of Ficin S was observed at pH 8.0 as shown in Fig. 7 and the enzyme was most active at 60 °C. Ficin S was stable over the pH range 2–8 at 4 °C for 20 h and also stable at temperatures up to 60 °C at pH 7.0 for 30 min, but the enzyme was completely inactivated at 67 °C.

Effect of activators and inhibitors on the enzyme activity

To determine the effect of activators on the activity of Ficin S an excess amount of $Na_2S_4O_6$ in purified Ficin S was removed by gel-filtration on a Sephadex G-50 column (1.65 cm × 90 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). As shown in Table II, cysteine, mercaptoethanol. and thioglycolic acid at 10^{-2} M, were found to completely activate the enzyme, but these reagents at 10^{-3} M and KCN exhibited a poor effect. On the other hand, $Na_2S_2O_3$ and ascorbic acid had no effect on the activity.

In order to examine the effect of inhibitors on the activity of the enzyme, Ficin S

TABLE II

EFFECT OF ACTIVATORS AND INHIBITORS ON ACTIVITY OF FICIN S FROM *FICUS CARICA* VAR. HŌRAISHI

Activity was measured by the method of Kunitz [8] in the presence of 2% casein and various concentration of activators and inhibitors at pH 7.0 and 37 °C for 30 min.

Activator	Relative activity (%)*		Inhibitor	Remaining activity (%)	
	10^{-2} M	10^{-3} M		10^{-3} M	10^{-4} M
Cysteine	99	18	none	100	—
Mercaptoethanol	79	20	$HgCl_2$	0**	3.5***
Thioglycolic acid	83	8.0	*p*-chloromercuribenzoate	0**	76***
KCN	1.8	0	$Na_2S_4O_6$	3.6	19
Ascorbic acid	0	0	H_2O_2	0	23
$Na_2S_2O_3$	0	0	I_2	0	50
			iodoacetic acid	0	13
			N-bromosuccinimide	32	79

* 100% activity was attained in the presence of 0.025 M cysteine.
** 10^{-5} M.
*** 10^{-6} M.

was activated by a 5000-fold molar excess amount of mercaptoethanol at pH 7.0 and 37 °C for 30 min. The activated enzyme was separated from mercaptoethanol by gel-filtration on a Sephadex G-50 column (1.65 cm × 90 cm) equilibrated with 0.1 M acetate buffer (pH 3.8). The effluent was adjusted to pH 7.0 by 0.1 M Na_2HPO_4.

As shown in Table II, $HgCl_2$ and *p*-chloromercuribenzoate showed a marked inhibitory effect and I_2, H_2O_2, $Na_2S_4O_6$ and iodoacetic acid also showed inhibition at 10^{-3} and 10^{-4} M, but inhibition by *N*-bromosuccinimide was not so strong as the above reagents. These results suggest that the enzyme is clearly a typical SH-enzyme.

Hydrolysis ratio of casein

The time course of the hydrolysis of casein by Ficin S is shown in Fig. 8. The hydrolysis ratio of casein was estimated to be 13% at 48 h. To know the comparative

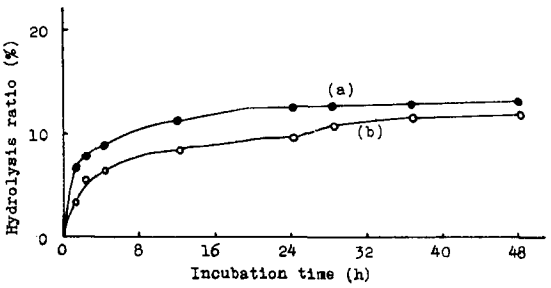

Fig. 8. Hydrolysis of casein by Ficin S (a) and Ficin A + Ficin S (b) Casein was hydrolyzed by Ficin S (3.3 units/g casein) in the presence of 0.025 M mercaptoethanol at pH 7.0 for 48 h and the time course of the hydrolysis was followed by the formol titration method [10]. The value was expressed as 100% in the case that casein was hydrolyzed by 6 M HCl at 110 °C for 48 h. To determine the cross reaction between Ficin A and Ficin S, casein was preliminarily hydrolyzed by Ficin A (1.67 units/g casein) for 24 h and then the same amount of Ficin S was added and the hydrolysis was continued for another 24 h.

specificity of Ficin S, casein substrate was preliminary hydrolyzed by 1.67 units/g casein of Ficin A for 24 h at 37 °C and then the same amount of Ficin S was added to the reaction mixture, the hydrolysis was performed for another 24 h. As in the result in Fig. 8, the hydrolysis ratio was less than that of Ficin S only. This result suggests that the comparative specificity of Ficin S is similar to that of Ficin A. One of the authors [17] reported that the comparative specificity of Ficin A, B, C and D resembled each other. Accordingly, it is considered that the comparative specificity of Ficin S, A, B, C and D are similar with each other.

DISCUSSION

In previous papers [4–6, 18], it has been reported that four proteinases, i.e. Ficin A, B, C and D which are natively contained in the latex of *Ficus carica* var. Hōraishi, have been isolated from the latex, and that the multiplicity of the enzymes has been discussed with regard to their enzymatic, chemical and physical properties and their stability and physiological activity.

In this report, in order to further clarify the multiplicity of the proteinases, sugar-containing proteinase (Ficin S) was purified to homogeneity by CM-cellulose and CM-Sephadex C-50 from the latex and crystallized.

Ficin S is considered to be a glycoprotein because the mobility of the protein band is equal to that of sugar in disc electropohresis, and because the sugar content (4.8 %) of Ficin S was constant before and after isoelectric focusing. On the other hand, sugar was not detected in Ficin A, B, C and D by staining with periodate–Schiff reagent [14] following disc electrophoresis and the sugar contents of Ficin A, B, C and D were negligible with phenol–H_2SO_4 method [16]. This result is different from the report [19] that all three ficin components from *Ficus glabrata* contained 2–5 sugar residues per molecule enzyme and that the sugar composition of the components differed.

Some enzymatic properties of Ficin S are listed in Table III in comparison

TABLE III

PROPERTIES OF FICIN S, A, B, C AND D FROM *FICUS CARICA* VAR. HŌRAISHI

Property	Ficin S	Ficin A[a]	Ficin B[a]	Ficin C[a]	Ficin D[a]
Spec. act. (units/mg)					
casein	1.8	2.0	2.8	3.7	5.7
Tos-Arg-OMe($X10^{-2}$)[b]	2.4	2.4	12.3	2.5	2.6
Optimum pH	8.0	7.5	7.0	7.0	7.5
Optimum temperature (°C)	60	65	60	78	55
pH-stability	2			8	
Thermal stability (°C)[c]	67	67	63	80	56
Hydrolysis ratio of casein (%)	13	13	18	17	16
Activators (10^{-2} M)[d]					
cysteine	99	103	96	98	98
mercaptoethanol	80	74	75	90	82
Inhibitors (10^{-6} M)[e]					
$HgCl_2$	3.5	2.0	3.7	3.5	3.0
p-chloromercuribenzoate	76	74	30	9.6	8.4
Isoelectric point (p*I*)[f]	9.1	8.3	10.2	>10.2	≫10.2
Molecular weight ($X10^4$)[g]	2.6	2.4	2.4	2.6	2.6
Sugar content (%)[h]	4.8	0	0	0	0

[a] The data were cited from refs 4 and 5.
[b] Measured by the method of Hummer [9].
[c] Inactivation temperature at pH 7.0 for 30 min.
[d] The activity attained in the presence of 0.025 M cysteine was taken as 100%.
[e] Remaining activity (%).
[f] Measured by isoelectric focusing and polyacrylamide gel electrophoresis [5].
[g] Measured by sodium dodecylsulfate–polyacrylamide gel electrophoresis [15].
[h] Measured by phenol–H_2SO_4 method [16] using glucose as the standard.

with those of Ficin A, B, C and D. Ficin S is similar to the other enzymes in optimum pH and temperature, specific activity, pH stability, behavior with activators and inhibitors, molecular weight and comparative specificity.

From these results, it is suggested that Ficin S differs only in sugar content and isoelectric point from the others. The isolation and characterization of glycopeptide of Ficin S are now in progress in our laboratory.

ACKNOWLEDGEMENTS

We wish to thank Miss E. Kuriyama for the preparation of the microphotograph of Ficin S.

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