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Studies on Proteinases from *Ficus carica* var. Hōraishi. VI. Immunochemical Comparison of Ficins A, B, C, D and S[1a,b]

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To clarify the multiplicity of proteinases, *i.e.* Ficins A, B, C, D, and S (EC 3.4.4.12) from *Ficus carica* var. Hōraishi, immunochemical properties of the enzymes were examined. Each enzyme used was proved to be homogeneous with immunoelectrophoresis. Antibodies were purified from rabbit antisera by ammonium sulfate fractionation, the use of Ficins immunoadsorbents and Sephadex G-200 gel-filtration. The relation of the enzymes was examined with Ouchterlony's double immunodiffusion, immunoelectrophoresis, cross-reaction among the enzymes on an immunoadsorbent column and inhibition of the caseinolytic activity of the enzymes by the antibodies.

The results from the experiments are as follows: (a) Ficins A and S were immunochemically identical and also Ficins B and C resembled to each other. (b) Ficins A and S, Ficin B, Ficin C, and Ficin D possessed common antigenic determinants at the rate of more than 38%, but the enzymes could be distinguished, in case that the relation of Ficin A and Ficin S be excepted.

From these results, it is suggested that the immunochemical similarity of the enzymes to Ficin A is in the order of Ficin A=Ficin S>Ficin B>Ficin C>Ficin D.

The existence of multiple forms of ficin has been well demonstrated in commercially available *Ficus glabrata* latex.[3–7] Williams, Glazer and Friedenson have purified ficin components from the dried latex of *Ficus glabrata* and characterized those properties.[4–6]

Previously, the authors have also reported that the latex from *Ficus carica* var. Hōraishi contains five proteinases (Ficins A, B, C, D and S) which differed in isoelectric point, thermal stability, chromatographic behavior and sugar content.[8–11]

In this paper, we report on the immunochemical properties of the enzymes in order to clarify the multiplicity of the enzymes. The results suggest that Ficins A, B, C, D and S can be distinguished except the relation of Ficin A and Ficin S, but that these enzymes share common antigenic determinants. The extent of similarity of the enzymes are also discussed.

Materials and Methods

Enzymes and Reagents——Ficins A, B, C, D, and S were purified from the latex of *Ficus carica* var. Hōraishi in conformity with the method of Sugiura and Sasaki.[8,11] Hammersten quality casein was a product

1) *a*) Part V: M. Sugiura and M. Sasaki, *Biochim. Biophys. Acta*, **350**, 38 (1974); *b*) This forms part CVI of "Studies on Enzymes" by M. Sugiura.
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3) V.C. Sgarbieri, S.M. Gupte, D.E. Kramer, and J.R. Whitaker, *J. Biol. Chem.*, **239**, 2170 (1964).
4) D.C. Williams and J.R. Whitaker, *Plant Physiol.*, **44**, 1574 (1969).
5) I.K. Jones and A.N. Glazer, *J. Biol. Chem.*, **245**, 2765 (1970).
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8) M. Sugiura and M. Sasaki, *Yakugaku Zasshi*, **91**, 457 (1971).
9) M. Sugiura and M. Sasaki, *Yakugaku Zasshi*, **93**, 63 (1973).
10) M. Sugiura and M. Sasaki, *Yakugaku Zasshi*, **93**, 338 (1973).
11) M. Sugiura and M. Sasaki, *Biochim. Biophys. Acta*, **350**, 38 (1974).

of E. Merck. Chromagel A-2 was obtained from Dojin-Yakkagaku Lab. Cyanogen bromide (BrCN) and agarose were purchased from Nakarai Chemicals Ltd. Sephadex G-200 was obtained from Pharmacia Fine Chemicals AB. The other chemicals used were of special or reagent grades.

Immunization Process——A 1% enzyme solution in physiological saline was emulsified with an equal volume of Freund's complete adjuvant (Iatron Lab., Tokyo). About 2 ml of this emulsion was injected intracutaneously in all foot pads and various positions on the back of a rabbit weighing 2.5—3.0 kg. As from the 30th day later, the same solution was injected by intra- or subcutaneous route once a week during 2 to 3 weeks, and the whole blood was collected 3 days after the last injection. The antiserum was incubated at 56° for 30 min and stored at 4°.

Purification of Antibody——The IgG fraction was separated from the antiserum by three successive precipitation with ammonium sulfate (the first precipitation at 40% saturation, and two consecutive ones at 33% saturation.[12]) Antibody was isolated from the IgG fraction by the use of Ficin immunoadsorbent which had been prepared by binding the enzyme to Chromagel A-2 according to the method of Axen *et al.*[13,14]) The adsorbed antibody was eluted with 0.17 M glycine-HCl buffer at pH 2.3 in a cool place, immediately neutralized and then dialyzed against borate saline, pH 7.9 (0.15 M NaCl containing 0.02 M borate buffer). The eluted antibody was further purified to remove macroglobulin by gel chromatography of Sephadex G-200 using borate saline.

Table I. Purification of Antibodies from Rabbit Anti-Sera

Procedure	Anti-Ficin A mg (%)[a)]	Anti-Ficin S mg (%)[a)]	Anti-Ficin B mg (%)[a)]	Anti-Ficin C mg (%)[a)]	Anti-Ficin D mg (%)[a)]
Anti-serum	2720 (100)	3360 (100)	4690 (100)	3030 (100)	3700 (100)
$(NH_4)_2SO_4$ fractionation (40% satn., 33% satn. 2 times)	457 (17)	732 (22)	1740 (37)	1070 (35)	1060 (29)
Ficins immunoadsorbent	128 (5)	141 (4)	584 (12)	352 (12)	450 (12)
Sephadex G-200 gel-filtration	51 (2)	111 (3)	505 (11)	270 (9)	313 (9)

a) the yield of antibody protein was expressed as percentages of the amount of anti-serum

Table I shows the purification of antibodies. The yields of anti-Ficins A and S were 2—3%, and those of anti-Ficins B, C, and D were 9—11%. Protein concentration was determined according to the method of Lowry[15]) using a bovine serum albumin (Daiichi Pure Chemicals Co. Ltd.) as standard in the course of antibody purification and determined from the ultraviolet (UV) adsorbance at 280 nm for IgG ($E^{1\%}_{1cm}=14.6$[16]).

Enzyme Assay——Caseinolytic activity was measured by the method of Kunitz[17]) in the presence of 0.5% casein and 0.025 M cysteine at pH 7.9 and 37°.

Inhibition Assay——The extent of inhibition of the caseinolytic activity was studied in the same manners as described in the enzyme assay except the preincubation of enzyme and antibody. The enzyme and antibody were mixed, allowed to stand for 40 min. at 37° and then 30 min. in a cool place. An aliquot of this incubation mixture was used for assays. The IgG fraction from normal serum did not cause inhibition.

Double Immunodiffusion and Immunoelectrophoresis——The micromethod of Ouchterlony technique[18]) was employed for the double immunodiffusion. A solution of 1.5% agarose in 0.15 M NaCl was poured onto a glass plate (2.6×7.5 cm) and allowed to make 1 mm thick layer jelly. The wells of 2 mm in diameter were filled with the antigens (enzymes) and antibodies, and the plates were kept in a humid chamber for 5 hr at 4°. Thereafter the precipitin lines were inspected directly or by staining with 1% Amido Schwarz 10B after the elimination of soluble proteins.

Immunoelectrophoresis was carried out in 1.5% agarose, 0.05 M veronal buffer, pH 7.5, for 60 min. on a power of 3 V/cm at 20°. The development by antibody was carried out at 4° for 18 hr.

12) R. Arnon and E. Shapira, *Biochemistry*, **6**, 3942 (1967).
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17) M. Kunitz, *J. Gen. Physiol.*, **30**, 291 (1947).
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Results

Immunoelectrophoresis of Ficins A, B, C, D and S

Immunochemical purity of the enzymes was examined by immunoelectrophoresis. As shown in Fig. 1, each of these enzymes formed one precipitin line reacted with the mixture of IgG fractions against the enzymes and there was no cross-contamination. The mobility of the enzymes toward cathode was in the order of Ficin D>Ficin C>Ficin B>Ficin S=Ficin A.

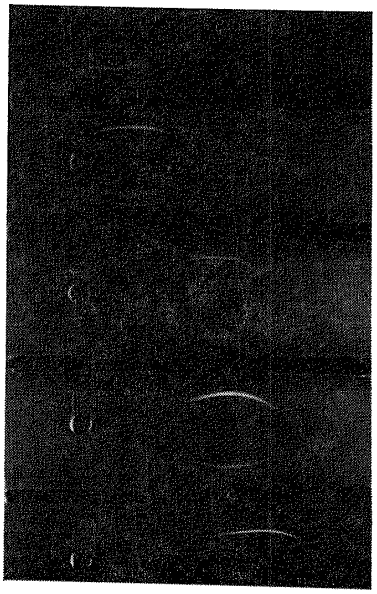

Fig. 1. Immunoelectrophoresis of Ficins A, B, C, D and S

The enzymes (1 mg/ml, 20 μl) were subjected to electrophoresis in 0.05M veronal buffer (pH 7.5) at 3 V/cm and 20° for 1 hr and then each trough was filled with the mixture of anti-Ficins IgG fractions (each 4.6 mg/ml, 70 μl). Immunodiffusion was done at 4° for 18 hr.

Cross-reaction among Ficins A, B, C, D and S

a) Double Immunodiffusion——Fig. 2 shows the double immunodiffusion of Ficins A, B, C, D and S with antienzymes. anti-Ficins antibodies were in the center wells and the enzymes were arranged peripherally so that each of them could be compared to the others. Ficins A, B, C, D and S formed crossreacting precipitin lines with anti-Ficins B, C, D and S aside from homologous enzyme-antienzyme systems. But anti-Ficin A reacted only to Ficins A and S to yield one merging precipitin line. From these results, it is considered that Ficins A and S are immunochemically identical, but that Ficins A and S, Ficin B, Ficin C and Ficin D are not, although all the enzymes possess common antigenic determinants.

b) Immunoelectrophoresis——Immunoelectrophoresis was also performed to detect the cross-reactivity of the enzymes with anti-enzymes. As shown in Fig. 3, all the wells were filled with the mixture of the enzymes, and after electrophoresis each trough was applied with one antibody. The results were almost identical with those of double immunodiffusion. Ficins A and S reacted with all the antibodies to yield fused precipitin lines indicating that Ficins A and S are almost identical. Anti-Ficin A reacted only to Ficins A and/or S and formed a fused precipitin line, but anti-Ficins B, C, D and S cross-reacted to every enzyme and formed precipitin line except for homologous enzyme-antienzyme systems. Anti-Ficin S reacted to all the enzymes to yield two fused precipitin lines which belong to Ficins A, S and Ficins B, C, D, respectively. Anti-Ficin D reacted to Ficins A, B, C and S to yield two fused precipitin lines which belong to Ficins A, S and Ficins B, C, D, respectively, indicating a spur from Ficins B and C over Ficins A and S. These results suggest that Ficins A and S cannot be immunochemically distinguished, while Ficins A and S, Ficin B, Ficin D can be distinguished respectively.

From the results of the double immunodiffusion and the immunoelectrophoresis, antigenic capacity of the enzymes is summarized below. The immunoreactivity of Ficins A and S to anti-Ficin A is equivalent. The immunoreactivity of the enzymes to anti-Ficin S is in the order of Ficin A=Ficin S>FicinD≧Ficin C≧Ficin B, to anti-Ficin B is in the order of Ficin B>Ficin C>Ficin D=Ficin A=Ficin S, to anti-Ficin C is in the order of Ficin C>Ficin B>Ficin D=Ficin A=Ficin S and, to anti-Ficin D is in the order of Ficin D>Ficin B=Ficin C≧Ficin S=Ficin A.

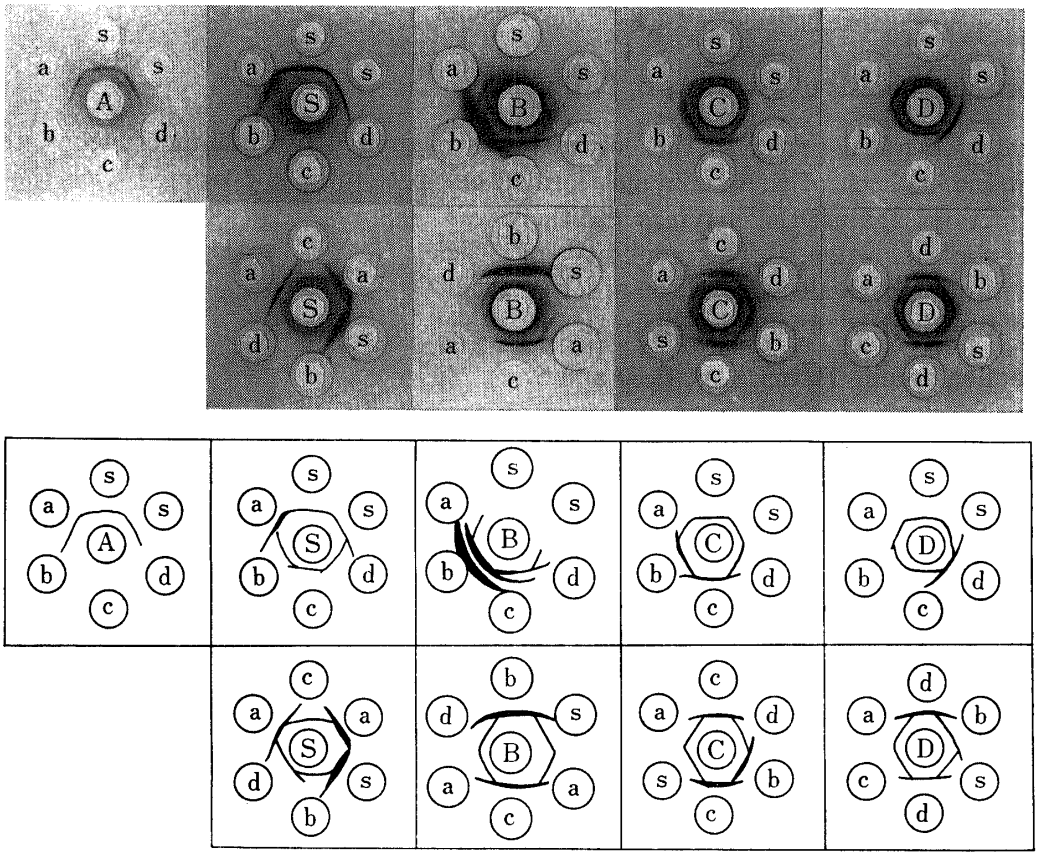

Fig. 2. Cross-Reactions among Ficins A, B, C, D and S in Agarose Gel

The antibodies (4 mg/ml, 7 μl) were placed in the center wells. Homologous and heterologous enzymes (125 μg/ml, 7μl) were put in alternate outer wells. A,anti-Ficin A; B, anti- Ficin B; C, anti-Ficin C; D, anti-Ficin D; S, anti-Ficin S; a, Ficin A; b, Ficin B; c, Ficin C; d, Ficin D; s, Ficin S. Immunodiffusion: 5 hr, 4°.

Cross-reaction among Ficins A, B, C, D and S on an Immunoadsorbent Column

Visible and invisible cross-reactions among the enzymes were examined using an immunoadsorbent column. A constant amount of each antibody was applied to a column with Chromagel-insolubilized enzyme. On a homologous system, more than 93% of the antibody was adsorbed with the exception of anti-Ficin A (73%). When the normal IgG was passed through the same column, 2—8% was trapped by nonspecific adsorption. The amount of nonspecific adsorption was thus substracted from both the homologous and heterologous adsorption values, and the net cross-reactions of the heterologous systems were calculated as percentages of those of the homologous systems and summarized in Table II.

The cross-reaction rate of Ficin S to anti-Ficin A was 100%, and those of Ficins B, C and D were 61—67%, but Ficins B, C and D did not form precipitin lines with anti-Ficin A in gel-diffusions. It can be assumed that Ficins B, C and D react with anti-Ficin A to form a soluble complex. Ficin A cross-reacted with anti-Ficin S at a rate of 98%, and cross-reaction rates of the enzymes to anti-Ficin S decreased in the order of Ficin S$\geqq$Ficin A$>$Ficin B$>$Ficin C$>$Ficin D. Anti-Ficin B cross-reacted to Ficin C at a rate of 91% and anti-Ficin C also cross-reacted to Ficin B at a rate of 85%, the fact of which showed that Ficin B and Ficin C resembled to each other. Cross-reaction rates of the enzymes with anti-Ficin D were in the order of Ficin D$>$Ficin C$=$Ficin B$>$Ficin A$>$Ficin S. Cross-reaction rates of Ficins A and S, and those of Ficins B and C also resembled, respectively. From these results, it is observed that the similarity between the enzymes and Ficin A is in the order of Ficin A$=$Ficin S$>$Ficin B$>$Ficin C$>$Ficin D. Adversely, the similarity between the enzymes and Ficin D is in the order

of Ficin D>Ficin C>Ficin B>Ficin S= Ficin A. This result corresponds to that of gel-diffusions.

Inhibition Experiment

Inhibition of caseinolytic activity of Ficins A, B, C, D and S by each antibody was measured at molar ratio of antibody to enzyme being 4.0. As shown in Table III, anti-Ficin A slightly inhibited the activity of Ficins A, B, C and S, whereas Ficin D was slightly activated by anti-Ficin A. This can be related to the fact that invisible cross-reactions among Ficins B, C and D to anti-FicinA occurred at a rate of 61—67%. Anti-Ficin S completely inhibited the activity of Ficins A and S. Ficins B and C were fairly inhibited by the antibody, but Ficin D was slightly activated. The activity of Ficin B was inhibited by anti-Ficin B at a rate of 79%, and the inhibition rates of Ficins A, C and S were 47—57%, but Ficin D was only 16%. Ficin C was inhibited by anti-Ficin C at a rate of 88%, and the inhibition rates of Ficins A, B, D and S were 31—47%.

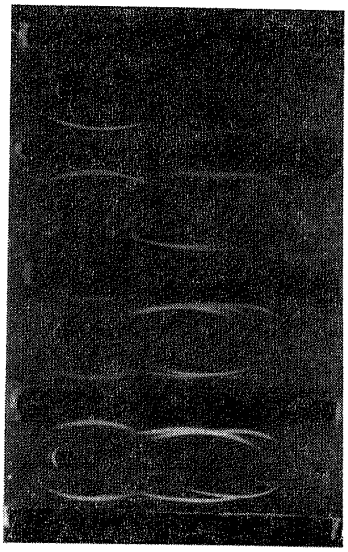

Fig. 3. Immunoelectrophoretic Demonstration of the Cross-Reactions among Ficins A, B, C, D and S by the Use of Each Antibody

Agarose plate was prepared with 1.5% agarose solution containing 0.05M veronal buffer, pH 7.5. The mixture of the enzymes (each 900μg/ml, 20 μl) was placed in the wells. After electrophoresis at 3V/cm for 1 hr, each antibody (4 mg/ml, 100 μl) was applied in the troughs as indicated and immunodiffusion was done at 4° for 18 hr.

Table II. Cross-Reactions among Ficins A, B, C, D and S on an Immunoadsorbent Column

Antibody	Cross-reaction (%)				
	Ficin A	Ficin S	Ficin B	Ficin C	Ficin D
Anti-Ficin A	100	100	61	67	61
Anti-Ficin S	98	100	74	71	52
Anti-Ficin B	47	39	100	91	47
Anti-Ficin C	39	38	85	100	53
Anti-Ficin D	61	53	78	75	100

Figures represent the percentage cross-reaction when the respective monospecific antienzyme was applied on the insolubilized enzyme column. The column was 0.9×3.5 cm and contained 6 to 8 mg of attached enzyme. The amount of antibody used for one trial was 2.5 to 3 mg.

Only Ficin D was strongly inhibited by anti-Ficin D, but the inhibition of the other enzymes was much lower than that of Ficin D. From the results, it is assumed that Ficin A is, antigenically, considerably different from Ficin D. This conforms the results of cross-reaction on an immunoadsorbent column.

Discussion

Immunological cross-reaction of proteins may be taken as indication of structual resemblance between the proteins in question,[19] in other words for the presence of similar antigenic

19) E.A. Kabat, "Experimental Immunochemistry," 2nd. ed., O. Kabat and O. Mayer, Ed., C.C. Thomas, Springfield, Ill., 1961, p. 405.

Table III. Inhibition of Caseinolytic Activity of Ficins A, B, C, D and S by Antibodies

Antibody	Inhibition (%)				
	Ficin A	Ficin S	Ficin B	Ficin C	Ficin D
Anti-Ficin A	15	5	8	1	$-2^{a)}$
Anti-Ficin S	99	97	88	74	$-3^{a)}$
Anti-Ficin B	57	47	79	56	16
Anti-Ficin C	31	34	47	88	32
Anti-Ficin D	12	1	7	27	97

a) activation

Immunological reaction was achieved at pH 7.9. The reaction mixture was allowed to stand for 40 min at 37° and then 30 min in the cold. Enzymatic activity of the resulting mixture was assayed under standard conditions. Antibody/enzyme was 4.0 (molar ratio). The residual activity attained in the presence of normal IgG was taken as 100%. The amount of the enzymes was 4 to 10 μg/ assay tube.

determinants on the cross-reacting antigen. The implication is that these determinants have the capacity to react with antibodies elicited by either protein. Such cross-reactions have been demonstrated in the case of several proteins including papain (EC 3.4.4.11),[20] bovine trypsin (EC 3.4.4.4) and α-chymotrypsin (EC 3.4.4.5)[21] and stem bromelain (EC 3.4.4.24) and fruit bromelain (EC 3.4.4.24).[22]

In the course of studying the proteinases from *Ficus carica* var. Hōraishi,[8–11] the authors have reported that Ficins A, B, C and D are similar in molecular weight, optimum pH, pH-stability, N-terminal amino acid, amino acid composition, but different in thermal stability, isoelectric point, tryptic finger print and affinity to CM-cellulose, and that only Ficin S is the glycoprotein among the enzymes. In order to elucidate the multiplicity of Ficins A, B, C, D and S, immunochemical properties of the enzymes were examined.

Anti-Ficin A reacted to only Ficins A and S to give a fused precipitin line in double immunodiffusion and immunoelectrophoresis, but as shown in Table II, cross-reaction rates of Ficins B, C and D to anti-Ficin A were 61—67%. This discrepancy can be explained that anti-Ficin A reacted to Ficins B, C and D to form a soluble complex. Anti-Ficin S reacted to Ficins A and S to yield a fused precipitin line and also with Ficins B, C and D to give cross-reacting precipitin lines in double immunodiffusion and immunoelectrophoresis. The cross-reaction rate of Ficin A to anti-Ficin S and that of Ficin S to anti-Ficin A were 98% and 100%, respectively, whereas anti-Ficin A showed lower inhibition of the enzymes than anti-Ficin S in the experiments. This dissimilarity of anti-Ficins A and S may be related to the results that anti-Ficin A contains soluble complex forming antibodies at a rate of 61—67%, because the order of the similarity of the enzymes to Ficin A judging from cross-reaction rate and that obtained from inhibition experiments, fairly corresponded to the order of the antigenic capacity of the enzymes from immunodiffusions. It is interesting that, although Ficins A and S are immunochemically indistinguished, Ficin S is a glycoprotein, but Ficin A is not. The relation of these enzymes will be further elucidated with aforementioned problems, in the light of chemical properties of Ficins A and S.

The cross-reaction rate of Ficin B to anti-Ficin C and that of Ficin C to anti-Ficin B were 85% and 91%, respectively and those of Ficins B and C to anti-Ficins A, S and D were similar in each other. It is considered that Ficin B is similar to Ficin C. Furthermore, the order of antigenic capacity of the enzymes detected by immunodiffusions, the order of the similarity of the enzymes judging from cross-reaction rate and that judging from inhibition experiments were fairly comparable each other.

20) R. Arnon and E. Shapira, *Biochemistry*, **7**, 4196 (1968).
21) M.M. Sanders, K.A. Walsh, and R. Arnon, *Biochemistry*, **9**, 2356 (1970).
22) S. Iida, M. Sasaki, and S. Ota, *J. Biochem.* (Tokyo), **73**, 377 (1973).

From these results, it is suggested that Ficins A, B, C, D and S possess common antigenic determinants, and that Ficins A and S are immunochemically indistinguished, and that Ficin B is similar to Ficin C, although Ficins A and S, Ficin B, Ficin C and Ficin D are different in each other, and that the immunochemical similarity of the enzymes to Ficin A is in the order of Ficin A=Ficin S>Ficin B>Ficin C>Ficin D.