

Biological Significance and Localization of Antigenic Determinant Common to Thiol Proteases of Plant Origin

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The localization of the common antigenic determinant among four kinds of thiol proteases, papain [EC 3.4.22.2], ficin [EC 3.4.22.3], stem bromelain [EC 3.4.22.4], and fruit bromelain [EC 3.4.22.5] was investigated by estimating the immunological cross-reaction rate and the inhibition capacity of cross-reacting and noncross-reacting antibody species separable by means of enzyme immunoabsorbent. The existence of the common antigenic determinant was demonstrated by isolating a particular antibody population which could combine with all four enzyme immunoabsorbents. However the distribution of common regions between each pair of proteases was not identical. The anti-stem bromelain antibody cross-reacting with fruit bromelain was found to inhibit efficiently the catalytic activities of not only stem and fruit bromelains but also papain and ficin using benzoyl-L-arginine ethyl ester as a substrate. A comparison of the inhibition capacity between antibody species common to two enzymes and that common to three enzymes demonstrated that antibody common to the larger number of enzymes inhibited the catalytic activities of all four kinds of enzymes more markedly. It was concluded from these results that immunological cross-reaction made it possible to detect the conformational homology among four kinds of ontogenetically and phylogenetically differentiated thiol proteases, and that this homology did involve the active site and its proximate regions, suggesting that such regions related to the enzyme function have been best conserved in the long evolutionary process of this enzyme group, maintaining their similar proteolytic functions.

In a previous paper (1) the immunological cross-reaction between stem bromelain [EC 3.4.22.4] and fruit bromelain [EC 3.4.22.5] was studied. These enzymes have been differentiated ontogenetically in the pineapple plant. This pair of enzymes cross-reacted 20

–32% with each other producing a visible enzyme-antienzyme complex, and the cross-reacting antibodies separated from both anti-stem and fruit bromelain antisera suppressed the catalytic activities of these enzymes far more markedly than the noncross-reacting antibodies. Thus, it was concluded that the antigenic determinant common to the enzyme was located mainly in the proximity of the active

Abbreviations used in this paper are listed in the "APPENDIX."

sites of the enzymes. A similar example was presented by Arnon and Shapira (2, 3) with the thiol proteases papain [EC 3.4.22.2] and chymopapain [EC 3.4.22.6] derived from papaya latex. It was found more recently that the above cross-reaction between stem and fruit bromelains could be extended further to other thiol proteases, papain, and ficin [EC 3.4.22.3] from papaya and fig latices. However in the case of cross-reaction between papain and ficin, only one direction of enzyme-antienzyme system (ficin with anti-papain) was found to be precipitable, with a cross-reacting rate of 18%. In contrast, cross-reactions of bromelains with papain or ficin could not be detected at all by the usual cross-precipitation technique in the test tube or in agar. Such invisible cross-reactions were determined by estimating the cross-reacting antibody adsorbed on a heterologous enzyme immunoabsorbent column. Table I (4) shows the cross-reaction rate between four kinds of proteases (parentheses designate precipitable systems). As shown, most of the heterologous enzyme-antienzyme systems with a cross-reacting rate below 10% are nonprecipitable systems.

The aim of this paper is to establish the topographical relationship of the functional region and the antigenic determinant common not only to pairs of proteases but also to the four kinds of proteases, and to discuss the biological significance of this conformational homology.

TABLE I. Cross-reaction on immunoabsorbent columns (4). Figures represent the percentage cross-reaction when the antienzyme antibody was applied to the enzyme immunoabsorbent column. Parentheses indicate precipitable enzyme-antienzyme systems.

	Stem bromelain	Fruit bromelain	Papain	Ficin
Anti-stem bromelain	(100)	(20)	3	6
Anti-fruit bromelain	(32)	(100)	2	4
Anti-papain	3	10	(100)	(18)
Anti-ficin	2	6	26	(100)

MATERIALS AND METHODS

Materials—Stem bromelain (fraction 6) (5) was prepared in our laboratory from crude bromelain (lot, A-VII-8) obtained from Jintan Dole Company, Osaka. Fruit bromelain (A) (6) was purified from commercially purchased pineapple fruit. Papain (twice crystallized, lot 119 B-5581) and ficin (twice crystallized, lot 119 B-4750) were preparations from Worthington Biochemical Co. and Sigma Chemical Co., respectively. Hammarsten-quality casein was a product of E. Merck. α -N-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) was obtained from Protein Research Foundation, Minoh, Osaka. Chromagel A-2 (100–150 mesh) was purchased from Dojin-Yakkagaku Laboratories. DEAE-cellulose was obtained from Seikagaku-Kogyo Co., Ltd. Freund's complete adjuvant was from Difco Laboratories.

Purification of Specific Antibody—Enzyme-specific antisera were prepared by immunizing rabbits with each enzyme emulsified with Freund's complete adjuvant. IgG fraction was separated from the antisera by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography. Specific antibodies were isolated from the IgG fractions by the use of homologous enzyme immunoabsorbent prepared by linking the enzyme to Chromagel A-2. Further separation of each specific antibody was achieved by applying the antibody to cross-reacting heterologous enzyme immunoabsorbent column. The details of the conditions used in these procedures were described in previous papers (1, 7).

Estimation of Enzymic Activity and Inhibition Capacity—The catalytic activity of enzymes for both casein and BAEE and the inhibition capacities of the antibody species to the enzymes were estimated by the same procedures as before (1, 7).

RESULTS

Immunological Cross-reaction between Papain and Ficin—In order to elucidate the localization and extent of the common antigenic determinant, an initial attempt was made

to characterize the cross-reaction between papain and ficin for comparison with results previously obtained with bromelain-anti-bromelain systems (1). Four species of antibodies from anti-papain and anti-ficin IgG antibodies were separated, as illustrated in Fig. 1, using the methods adopted for the study of bromelains. Common antibodies (cFi(P) and cP(Fi)) were cross-adsorbed on partner enzyme columns, and were then dissociated with a low pH buffer (0.17 M glycine-HCl buffer, pH 2.3) at 2–4°, immediately followed by neutralization and complete dialysis against 0.02 M borate-buffered saline, pH 8.0. The other two species of noncross-reacting antibodies were collected and designated as nFi(P) and nP(Fi). The capacity of the four species of antibodies thus obtained to inhibit enzymic activity was estimated using both high- and low-molecular-weight substrates (casein and BAEE). Figures 2 and 3 show the results of quantitative inhibition assay. As expected, noncross-reacting antibodies nFi(P) and nP(Fi) did not cross-inhibit the catalytic activities of the heterologous enzymes, irrespective of the molecular size of the substrate used, but these antibodies were capable of inhibiting the homologous

enzymes to some extent. However, cross-reacting antibodies cFi(P) and cP(Fi) showed ability to inhibit not only homologous but also heterologous enzymes. The inhibition capacities of cross-reacting and noncross-reacting antibodies are summarized in Table II(A). In the homologous enzyme-antienzyme system the ratio of the inhibition capacity of cFi(P) (which accounts for 18% of the total anti-papain antibody population) to nFi(P) (corresponding to the remainder, 82%) was 5:6 when BAEE was used as a substrate. This means that the content of antibody able to inhibit papain activity is almost the same in the cross-reacting and noncross-reacting antibodies. On the other hand, in the ficin-anti-ficin system most of the antibody population directed to-

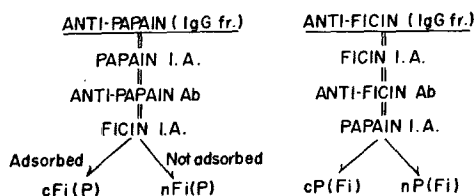


Fig. 1. Separation procedures for cross-reacting and noncross-reacting antibodies using papain and ficin immunoabsorbent columns. I.A.; immunoabsorbent. For other abbreviations, see the "APPENDIX." 1st step; anti-papain or anti-ficin IgG fraction was applied to homologous enzyme immunoabsorbent column to eliminate normal or inactive IgG. 2nd step; separation of cross-reacting and noncross-reacting antibodies by means of heterologous enzyme immunoabsorbents. The column sizes were 1.2×10 cm. The column contained 100 to 110 mg of enzyme, possessing the capacity to adsorb 150 to 200 mg of homologous antibody. A hundred milligrams of antibody was usually used for the separation of cross-reacting and noncross-reacting antibodies. All the procedures were carried out in the cold (2–4°).

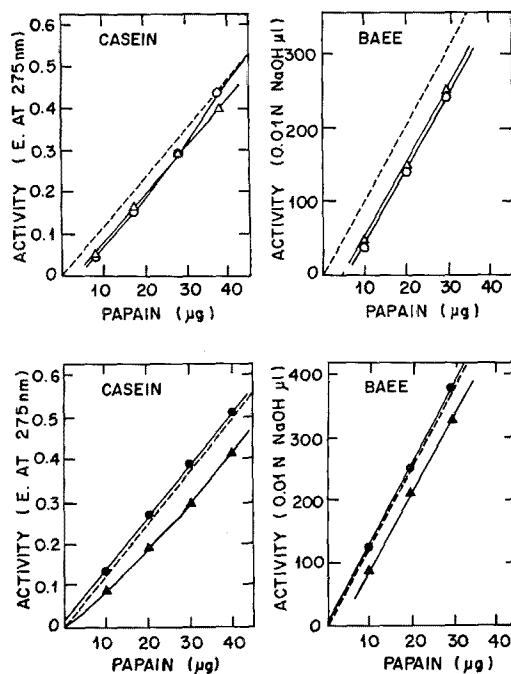


Fig. 2. Estimation of the inhibition capacities of four species of antibodies to papain. The amount of antibody used was 250 μ g. Dotted lines represent enzymic activity in the absence of antibody. Inhibition assay using casein as a substrate was carried out at 30°, pH 7.5. Inhibition assay using BAEE as a substrate was carried out at 25°, pH 6.0 using an autotitrator with 0.01 M NaOH (1, 7). Δ , cFi(P); $\circ$, nFi(P); $\blacktriangle$, cP(Fi); $\bullet$, nP(Fi). For abbreviations, see the "APPENDIX."

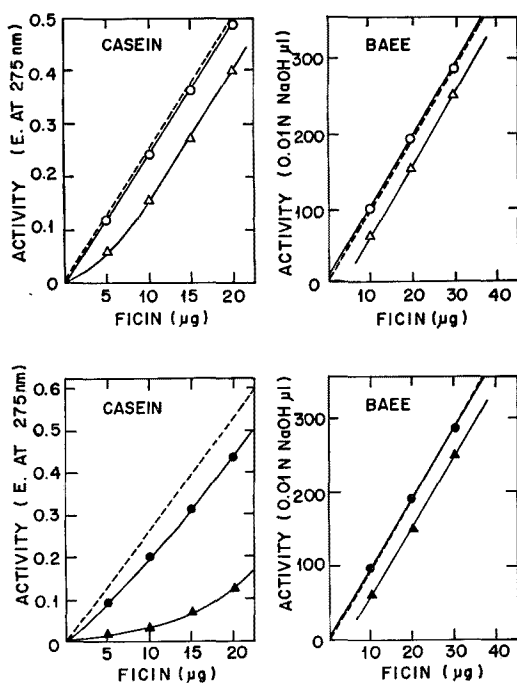


Fig. 3. Estimation of the inhibition capacities of four species of antibodies to ficin. The amount of antibody used was 250 μ g. Dotted lines represent enzymic activity in the absence of antibody. Inhibition assay using casein as a substrate was carried out at 30°, pH 7.5. Inhibition assay using BAEE as a substrate was carried out at 25°, pH 6.0, using an autotitrator with 0.01 M NaOH (1, 7). Δ , cFi(P); $\circ$, nFi(P); $\blacktriangle$, cP(Fi); $\bullet$, nP(Fi).

ward the active site region of the enzyme appears to be present in the cross-reacting antibody (cP(Fi)), because detectable inhibition with BAEE as a substrate was observed only when cross-reacting antibody cP(Fi) was used. Likewise, in homologous systems of bromelains and their antibodies both kinds of cross-reacting antibody species, namely cF(S) and cS(F) showed much higher inhibition capacity than the noncross-reacting antibodies, as shown in Table II (B) (1). From the results with these four sets of homologous enzyme-antienzyme systems [papain -cFi(P) and nFi(P), ficin -cP(Fi) and nP(Fi), stem bromelain -cF(S) and nF(S), and fruit bromelain -cS(F) and nS(F)], it seems reasonable to conclude that the cross-reacting antibodies are directed more to the active site region of enzymes as compared with the noncross-reacting antibodies.

Antibody Common to Four Kinds of Thiol Proteases—In order to verify the existence of a common antigenic determinant to all four enzymes, anti-stem bromelain antibody was first applied to the fruit bromelain immuno-adsorbent column (Fig. 4). The anti-stem bromelain antibody cross-reacting with fruit bromelain (cF(S)) amounted to 18–23% of the total. The cross-reacting antibody was then applied to the papain immuno-adsorbent column, and was separated into two species; anti-stem bromelain antibody cross-reacting with both fruit bromelain and papain (cFcP(S)) and antibody cross-reacting with fruit bromelain but not with papain (cFnP(S)). The

TABLE II(A). Inhibition capacities of cross-reacting and noncross-reacting antibodies using casein and BAEE as substrates—Enzyme-antienzyme systems between papain and ficin. Inhibition capacity represents the maximum amount of enzyme (μ g) which could be completely inhibited by a given amount of antibody (250 μ g). The values were calculated from the experimental results in Figs. 2 (A) and 2 (B) by extrapolating the enzymic activities in the presence of the antibody species to the abscissa. "Proportions of antibody" in the 2nd column are based on the data in Table I.

Antibody species	Proportions of antibody	Papain Inhibition capacity		Ficin Inhibition capacity	
		Casein (μ g)	BAEE (μ g)	Casein (μ g)	BAEE (μ g)
cFi(P)	18 (%)	4	5	4	4
nFi(P)	82	5	6	0.5	0
cP(Fi)	26	7	3	15	4
nP(Fi)	74	0	0	4	0

TABLE II(B). Inhibition capacities of cross-reacting and noncross-reacting antibodies using casein and BAEE as substrates—Enzyme-antienzyme systems between stem and fruit bromelains (1). Inhibition capacity represents the maximum amount of enzyme (μg) which could be completely inhibited by a given amount of antibody ($250\ \mu\text{g}$). "Proportions of antibody" in the 2nd column are based on the data in Table I.

Antibody species	Proportions of antibody	Stem bromelain Inhibition capacity		Fruit bromelain Inhibition capacity	
		Casein	BAEE	Casein	BAEE
cF(S)	20 (%)	9 (μg)	14 (μg)	10 (μg)	5 (μg)
nF(S)	80	13	6	1	1
cS(F)	32	10	18	23	25
nS(F)	68	2	2	27	7

cFcP(S) antibody was further applied to the ficin immunoabsorbent column to obtain antibody common to all four kinds of thiol proteases (cFcPcFi(S)). Anti-stem bromelain antibody not cross-reacting with fruit bromelain (nF(S)) was also applied to the papain immunoabsorbent column, and a small amount of antibody cross-reacting with papain (nFcP(S)) was isolated in addition to the antibody cross-reacting with neither fruit bromelain nor papain (nFnP(S)).

Inhibition Capacities of the Various Antibodies—The inhibition capacities of the various antibody species obtained above were estimated using BAEE and casein as substrates. The summarized data are listed in Tables III (A) and III(B). As shown in Table III(A), anti-stem bromelain antibody cross-reacting with fruit bromelain (cF(S)) inhibited papain and ficin as well as stem and fruit bromelains, even when a substrate of small molecular size,

BAEE, was used. However the antibody species cross-reacting with both fruit bromelain and papain (cFcP(S)) inhibited all four kinds of enzymes, including ficin more markedly. Moreover the fact that cFnP(S) antibody (prepared from cF(S) antibody by separating the antibody species cross-reacting with papain (see Fig. 4)) mostly lost its inhibition capacity indicates that cFcP(S) is an antibody species among cF(S) antibody that is more selectively

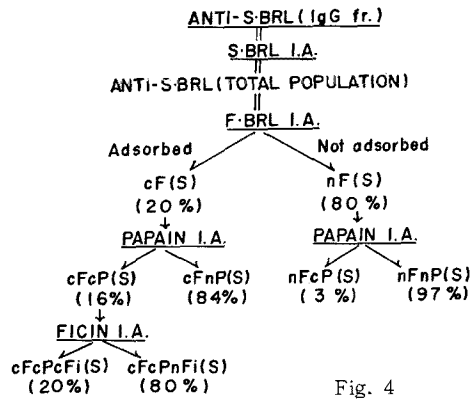


Fig. 4

Fig. 4. Separation of the anti-stem bromelain antibody population into several antibody species by means of cross-reacting enzyme immunoabsorbent columns. Pooled anti-stem bromelain antisera (350 ml) was used for this experiment. Anti-stem bromelain IgG fraction was prepared by repeated (twice) ammonium sulfate precipitation, followed by DEAE-cellulose column chromatography. The specific anti-stem bromelain antibody was purified on the homologous enzyme immunoabsorbent column. Further separation was carried out using three kinds of cross-reacting enzyme immunoabsorbents. The size of the columns was $1.2 \times 10\text{ cm}$. Each column contained 100 to 110 mg of bound enzyme. The adsorption capacities of these immunoabsorbent columns for homologous antibody were 150 to 200 mg. A hundred milligrams or less of antibody was applied for each step of separation. The proportions of cross-reacting and noncross-reacting antibodies were determined by estimating UV absorption. The nonspecific adsorption by normal rabbit IgG ranged from 0 to 2.5%. Thus, the percentage of noncross-reacting antibody was calculated as the proportion of noncross-reacting antibody to applied antibody corrected by subtracting nonspecific adsorption, and the remainder was regarded as cross-reacting antibody.

TABLE III(A). Inhibition capacity of various antibody species separated from anti-stem bromelain antibody with BAEE. Inhibition capacity was estimated in the same manner as in the experiments shown in Table II. The values represent the maximum amount of enzyme (μg) which could be completely inhibited by a given amount of various antibody species (250 μg).

Antibody species	Thiol proteases			
	Stem bromelain	Fruit bromelain	Papain	Ficin
cF(S)	14	5	3	11
cFcP(S)	31	26	10	12
cFnP(S)	0	0	1	0
nF(S)	6	0	5	8
nFcP(S)	13	2	8	14
nFnP(S)	0	2	1	8

TABLE III(B). Inhibition capacity of various antibody species separated from anti-stem bromelain antibody with casein. Inhibition capacity was estimated in the same manner as in the experiments shown in Table II. The values represent the maximum amount of enzyme (μg) which could be completely inhibited by a given amount of various antibody species (250 μg).

Antibody species	Thiol proteases			
	Stem bromelain	Fruit bromelain	Papain	Ficin
cF(S)	9	10	18	19
cFcP(S)	51	45	40	34
cFnP(S)	11	36	0	0
nF(S)	14	2	10	8
nFcP(S)	20	4	36	38
nFnP(S)	12	2	3	4

directed toward the active site region of the enzymes.

Another noteworthy phenomenon is the inhibition spectrum of the antibody species not cross-reacting with fruit bromelain; nF(S) and nFcP(S). As represented in Table III(A), some inhibition capacity toward stem bromelain, papain, and ficin, but not fruit bromelain,

still remained in these antibody species, and this phenomenon is more marked in nFcP(S) antibody. It seems probable from these data that a part of the common antigenic determinant exists in a position other than that which cF(S) antibody combines with, suggesting that such a common determinant does not completely overlap but is partly distinct in each case.

Table III(B) shows the inhibition capacity of the same antibody species used in the experiment shown in Table III(A) when a high-molecular-weight substrate, casein, was used. A typical size effect of the substrate appears in three enzyme-antienzyme systems; stem bromelain -cFnP(S) antibody, fruit bromelain -cFnP(S) antibody, and stem bromelain -nFnP(S) antibody. Among these three systems, the inhibition in the former two systems is probably due to steric hindrance by the direct attachment of the antibody to regions relatively close to the active site, because cFnP(S) is an antibody species selected as a common antigenic determinant of fruit bromelain, and in addition it accounts for a so small part of the antibody population (17%) of anti-stem bromelain antibody to form a complete lattice (aggregate) (7), while the inhibition in the last system seems to be mostly due to lattice formation, because of the large population of this nFnP(S) antibody (78%). Other systems which do not change their inhibition rate on alteration of the molecular size of the substrate are of the type in which antibody does not bind to the enzymes, or if it does bind, the site is apart from the active site and no lattice formation occurs. The systems of fruit bromelain with nF(S), nFcP(S), and nFnP(S) and the systems of papain with cFnP(S) and nFnP(S) are included in the former category, based on the preparation procedures used for this antibody group.

DISCUSSION

Immunological features of a pair of cross-reacting enzymes, papain and ficin, were studied initially and the results were compared with those for another pair of enzymes, stem and fruit bromelains (Tables II (A) and II (B)).

These data suggest that although the common antigenic determinants between a pair of enzymes generally involve the active site and its proximate regions, the extents of these common areas vary in different enzymes. A partial deviation of common antigenic determinants was demonstrated in an experiment using anti-stem bromelain antibody not cross-reacting with fruit bromelain (Table III(A)). Namely, the antibody of nF(S) was proved to contain a certain amount of antibody cross-reacting with papain (nFcP(S)), which has a distinctive inhibitory action on each of the enzymes except fruit bromelain. On the other hand, the facts that the antibody common to all four enzymes could be obtained by the successive application of anti-stem bromelain antibody to immunoadsorbent columns of the other three enzymes, and that the antibody common to at least two enzymes inhibits the other enzymes very markedly as well, suggest that there exists an area common to all four enzymes, and that this particular portion corresponds to the region around the active site. The proportions of cross-reaction between the two bromelains and papain or ficin are very small, as shown in Table I, representing the phylogenetic distances. However, the inhibition capacity of the anti-stem bromelain antibody cross-reacting with both fruit bromelain and papain is far larger than that of the antibody cross-reacting with only fruit bromelain. These results indicate that the antibody population selected by the larger number of isofunctional enzymes was more sharply directed toward the functional region of this enzyme group. The homology of the functional region among the thiol proteases could be extended further to another thiol proteases chymopapain, for this enzyme was found to have a common antigenic determinant with papain (2, 3). Consequently the phylogenetic and ontogenetic relationships of the thiol proteases having conformational homology is illustrated in Fig. 5. According to the review by Greenberg and Winnick (8), there are several other thiol proteases of plant origin. Among them are pinguinain [EC 3.4.99.18] from maya fruit (9), asclepain [EC 3.4.22.7] from milkweed latex (10), mexicanain [EC 3.4.99.14] from

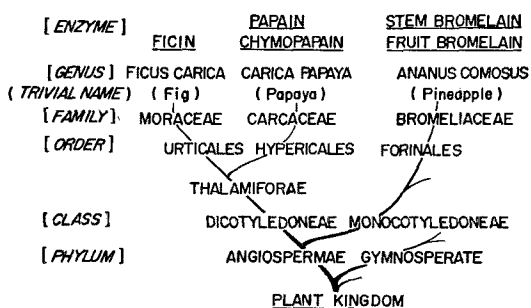


Fig. 5. Phylogenetic representations of immunologically cross-reacting thiol proteases. The immunological cross-reaction between papain and chymopapain was investigated by Arnon and Shapira (2, 3). Other relations were studied in our laboratory (1, 4). Cross-reactions between stem and fruit bromelains, and between papain and chymopapain showed precipitable reactions, while that between papain and ficin was precipitable in only one direction (see Table I). The cross-reactions between different classes of thiol proteases were all nonprecipitable reactions, indicating their phylogenetic distance.

cuaguayote leaves or fruit (11), euphorbain [EC 3.4.99.7] from caper spurge latex (12), and solanain [EC 3.4.99.21] from horsenettle fruit (13). These enzymes have a function similar to milk clotting as a common enzymic property. Although it is not yet known whether all these proteases possess a common antigenic determinant around the active site, the fact that all the thiol proteases so far studied exhibit homology in their functional regions strongly suggests the above possibility. Furthermore, whether a particular antibody capable of cross-inhibiting several thiol proteases of plant origin could also inhibit the catalytic activities of thiol proteases of animal and bacterial origin is of interest with regard to the phylogenetical relationships among living organisms. In contrast with the immunological studies, Husain and Lowe (14) tried to determine the amino acid sequences of the active site peptide from three kinds of thiol proteases; papain, ficin, and stem bromelain, by irreversible modification with 1,3-dibromo-[2-¹⁴C]acetone. In this comparative study, strong homology of amino acid sequence in the peptides was shown among these three thiol proteases. The superimposition of this

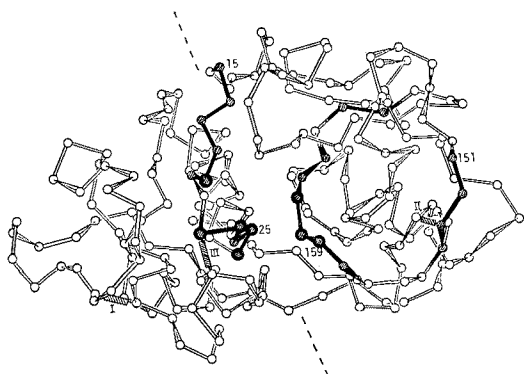


Fig. 6. Stereochemical illustration of homologous amino acids around the active sites of papain, ficin, and stem bromelain. Homologous amino acids in the active site peptides from papain, ficin, and stem bromelain (Husain and Lowe (14)) are superimposed on the three-dimensional structure of papain (Drenth, Jansonius, Koekoek, and Wolthers (16)). The shaded parts denote amino acids common to at least two proteases and those with thicker circles indicate amino acids common to three proteases. Amino acids 25 and 159 correspond to cysteine and histidine in the active site of papain.

homologous part onto the three-dimensional structure of papain obtained by X-ray analysis (15, 16) gives a more concrete stereochemical image of the common antigenic determinants (Fig. 6). The shaded parts denote amino acids common to at least two proteases and those with thick circles amino acids common to all three proteases. This chemical homology appears to fulfil the minimum indispensable condition to give the common antigenic structure around the active site. Direct evidence of the coincidence of chemical homology and conformational homology was demonstrated with α -chymotrypsin [EC 3.4.21.1] and anti-trypsin antibody system (17). In this experiment, a cross-reacting chymotrypsin peptide (consisting of 42 amino acids) was isolated by complexing with anti-trypsin antibodies, and identified as a peptide including the active site of this enzyme. It seems probable from these data that the immunologically detectable homology does indeed represent the existence of a chemical homology, but the reverse is not always necessarily the case, as was demonstrated for the relation between hen egg-white lysozyme [EC

3.2.1.17] and bovine α -lactalbumin. In this case, the two proteins have been shown to possess similar structural features (18) as regards molecular weight, amino acid sequence (19–21) (49 identical amino acids in a total of 129 amino acid residues of lysozyme and 123 amino acids of α -lactalbumin; 39–40% homology), and the number and position of disulfide bonds (four disulfide bonds each). A study of the three-dimensional structure also showed a high degree of similarity between them (22). In spite of this accumulation of evidence for structural similarity, no immunological cross-reaction could be observed (23, 24), nor were antibodies to α -lactalbumin capable of inhibiting the catalytic activity of lysozyme. Trypsin [EC 3.4.21.4] and α -chymotrypsin, as opposed to the case of lysozyme and α -lactalbumin, exhibited immunological cross-reaction with the reciprocal antibodies (17, 25), although the extent of chemical homology between their proenzymes (99 identical amino acids from a total of 245 amino acid residues of α -chymotrypsinogen (26, 27) and 229 amino acids of trypsinogen (28, 29); 40–43% homology) is not very different in degree from that between lysozyme and α -lactalbumin. The reason for the lack of cross-reaction between lysozyme and α -lactalbumin is presumably the change of conformation around the active site of α -lactalbumin, with a simultaneous alteration of the enzymic function: Lysozyme plays a role in cleaving the glycosidic linkage between N-acetylglucosamines, while α -lactalbumin participates in lactose synthesis with "A protein" (30, 31). This means that the three-dimensional structures of lysozyme and α -lactalbumin may not be homologous, but rather analogous or similar. The point to be stressed here is that immunological cross-reaction can distinguish such differences between conformational homology and analogy.

The thiol proteases used in the present experiments demonstrated immunological cross-reactions between enzymes not only from different orders but also from different classes of plant. Moreover, the immunological cross-reaction was always accompanied by notable cross-inhibition, even though the cross-reacting

rate was below 10%.

These results and the above considerations led us to the conclusion that the conformational homology at the active site region among the four kinds of thiol proteases has been preserved to maintain their similar functions of proteolysis, this being the biological significance of this common structure. How widely the homology is distributed in living organisms might represent a barometer of the significance of this conformation, and at the same time provides information about the historical origin of this fundamental structure.

The last problem to be discussed here is the immunogenicity of the active site itself. As Cinader pointed out (32), if the conformation of the active site is completely common to enzymes of the same group in all organisms, especially in immunized animals, the structure should not have immunogenicity because of the phenomenon known as immunological tolerance (33). However, provided that thiol proteases are intracellular enzymes and do not encounter the cell systems related to antibody production until the immunized animal is adult, then tolerance to this enzyme would not be set up in the immunesystems of the animals. Since thiol proteases ever found belong to intracellular enzymes, it seems probable that they have antigenicities as regards the conformation at the active site.

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APPENDIX

List of abbreviations used for enzymes and antibodies

S or S·BRL	Stem bromelain
F or F·BRL	Fruit bromelain
P or PAP	Papain
Fi or FIC	Ficin
cF(S)*	Anti-stem bromelain antibody cross-reacting with fruit bromelain
nF(S)	Anti-stem bromelain antibody not cross-reacting with fruit bromelain
cS(F)	Anti-fruit bromelain antibody cross-reacting with stem bromelain
nS(F)	Anti-fruit bromelain antibody not cross-reacting with stem bromelain
cP(Fi)	Anti-ficin antibody cross-reacting with papain

* Capital letters in parentheses designate starting IgG antibody specific to one kind of enzyme. ** The sequence in such nomenclature as cFcP represents the order in which antibody was applied to enzyme immunoadsorbents.

nP(Fi)	Anti-ficin antibody not cross-reacting with papain
cFi(P)	Anti-papain antibody cross-reacting with ficin
nFi(P)	Anti-papain antibody not cross-reacting with ficin
cFcP**(S)	Anti-stem bromelain antibody cross-reacting with both fruit bromelain and papain
cFnP(S)	Antibody not cross-reacting with papain among the anti-stem bromelain antibodies cross-reacting with fruit bromelain
nFcP(S)	Antibody cross-reacting with papain among the anti-stem bromelain antibodies not cross-reacting with fruit bromelain
nFnP(S)	Anti-stem bromelain antibody not cross-reacting with both fruit bromelain and papain
cFcPcFi(S)	Anti-stem bromelain antibody cross-reacting with fruit bromelain, papain, and ficin
cFcPnFi(S)	Antibody not cross-reacting with ficin among the anti-stem bromelain antibodies cross-reacting with fruit bromelain and papain