

Antigenic Determinant Common to Four Kinds of Thiol Proteases of Plant Origin

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The immunological cross-reaction is a very useful method for detecting molecular homology of the surface structures of related enzymes. Using this technique the homologies of papain [EC 3.4.4.10] and chymopapain [EC 3.4.4.11] (1), and of bovine trypsin [EC 3.4.4.4] and α -chymotrypsin [EC 3.4.4.5] (2) have been revealed. In a previous paper (3) we also reported a pair of cross-reacting thiol proteases, stem bromelain [EC 3.4.4.24], and fruit bromelain [EC 3.4.4.24]. This preliminary note describes another new pair of cross-reacting proteases, papain and ficin [EC 3.4.4.12], and the mutual cross-reactions between four kinds of thiol proteases (stem and fruit bromelains, papain, and ficin). However, nine of twelve heterologous reacting systems tested did not give detectable immunological precipitin reaction, because they were invisible (nonprecipitable) systems (see Table I). The existence of these invisible cross-reactions was demonstrated by the immunoabsorbent technique and by use of a Sephadex column as a molecular sieve. Papain (twice crystallized) and ficin (twice crystallized) were obtained from Sigma Chemical Co. Stem bromelain was purified from crude material in our laboratory (4), and fruit bromelain (A) was a generous gift from

Dr. S. Ota (5). The specific antibodies against each enzyme and cross-reacting common antibodies were isolated as described previously (3).

Double Immunodiffusion—Visible cross-reactions were revealed by double immunodiffusion in agar gel. As shown in Fig. 1, fruit bromelain, stem bromelain, and ficin each formed cross-reacting precipitin lines with anti-stem bromelain, anti-fruit bromelain, and antipapain antibodies besides from homologous enzyme-antienzyme systems. Thus with these four kinds of thiol proteases only three heterologous enzyme-antienzyme systems produced an insoluble complex, while the other heterologous systems gave a negative precipitin reaction. It is noteworthy that the papain-antificin system did not produce an insoluble complex, even though the reverse ficin-anti-papain system gave a visible precipitin reaction. A similar phenomenon was found by Sanders *et al.* (2) with the system of trypsin and chymotrypsin.

Cross-reactions Demonstrated on an Immunoabsorbent Column—The existence of an invisible cross-reaction was examined using immunoabsorbent. A constant amount of each specific antibody was applied to a column with Chromagel-insolubilized enzyme (3). With a homologous system more than 95 percent of the antibody was adsorbed, whereas when normal IgG was passed through the same column 2 to 3 percent was trapped

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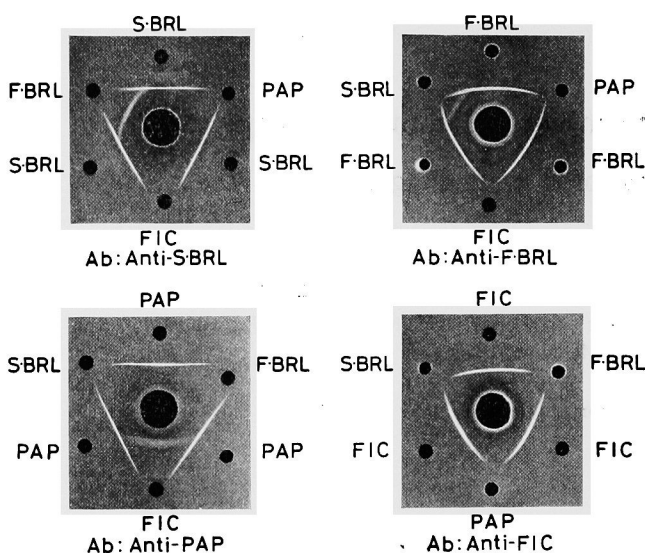


Fig. 1. Cross-reactions between four kinds of thiol proteases in agar gel. The specific antibodies indicated (3 mg/ml, 50 μ l) were placed in the center wells. Homologous and heterologous enzymes (1 mg/ml, 10 μ l) were put in alternate outer wells. S-BRL, stem bromelain; F-BRL, fruit bromelain; PAP, papain; FIC, ficin. Incubation time, 16 hr at 37°C.

TABLE I. Cross-reaction on an immunoabsorbent column. Figures represent the percentage cross-reaction when the respective monospecific antienzyme was applied on the insolubilized enzyme column. The column was 1.2 $\times$ 8 cm and contained 60 to 80 mg of attached enzyme. The amount of antibody used for one trial was 50 mg. Values in parentheses indicate those of visible cross-reaction 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)

by nonspecific adsorption. The adsorption of the heterologous systems varied from 4.5 to 35 percent. The amount of nonspecific adsorption was thus subtracted from both the homologous and heterologous adsorption values, and the net cross-reactions of the heterologous systems as percentages of those of the homologous systems were calculated and are summarized in Table I. Considering these data together with the result of immunodiffusion, it is assumed that the difference between visible and invisible cross-reactions is mainly due to the degree of cross-reaction: that in

the invisible systems ranged from 2 to 10 percent, while that in the visible systems was more than 10 percent. But this is not a strict distinction, as shown by the cross-reactions of the papain-antificin and chymotrypsin-antitrypsin (2) systems.

Interaction of Enzyme with Antienzyme in Heterologous Invisible Systems on a Sephadex Column—The population of cross-reacting antibody (antibody common with one kind of heterologous enzyme) was isolated from the total population of specific antibody to confirm the existence of cross-reactions in the invisible

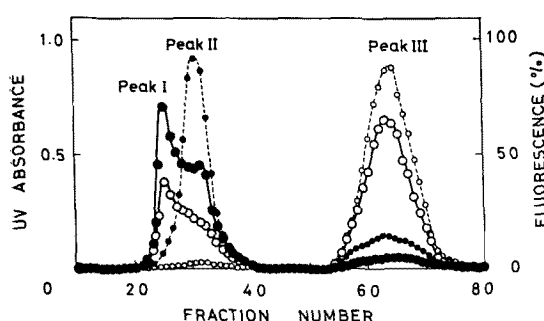


Fig. 2. Interaction of papain and anti-fruit bromelain cross-reacting with papain on a Sephadex G-150 column. Papain was labeled with fluorescein isothiocyanate (The binding number of fluorescein isothiocyanate per one molecule of modified enzyme was 0.19) (6). The antibody used in this experiment was prepared by applying anti-fruit bromelain on the insolubilized papain column, washing the column thoroughly with 0.02 M borate buffered saline pH 8.0, and dissociating the adsorbed antibody with 0.17 M glycine-HCl buffer pH 2.3 in the cold (4°C). Gel-filtration was performed at a flow rate of 4 ml/hr at 22°C. Column size, 1.0 × 100 cm. Fraction volume, 1.0 ml. Protein concentration was estimated at 280 nm, and fluorescence at 520 nm with an excitation wave length of 280 nm. Filled circles (—●—, ----) represent UV absorbance and open circles (—○—, ----) fluorescence. Large circles show results with the papain-anti-fruit bromelain system and small circles those with the papain-normal IgG system.

systems. This antibody and fluorescein-labeled enzyme were mixed, incubated at room temperature for 40 min, and then applied on a Sephadex G-150 column. The gel-filtration profile of the papain-anti-fruit bromelain system (which had the lowest rate of cross-reaction of 2 percent) is illustrated in Fig. 2. Peak II and peak III are the elution volumes of IgG and enzyme, respectively, which did not interact. Each component in a mixture of normal rabbit IgG and fluorescein-labeled papain was eluted separately in the position corresponding to its molecular size, but with a mixture of anti-fruit bromelain antibody common with papain and fluorescein-labeled papain, a new peak (peak I) appeared ac-

companied with transfer of the fluorescence. This indicates that papain and the anti-fruit bromelain antibody formed a soluble complex.

The tentative conclusions from this cross-reacting experiment coincide well with the phylogenetic relations between the four proteases. Papain and ficin, which are derived from plants in the same class (*dicotyledoneae*) but in different orders (papaya; *Hypericales* and fig; *Urticales*) cross-reacted 18–26 percent, and their visible reaction was observed only in one direction, whereas stem and fruit bromelains which are differentiated ontogenetically in the pineapple plant showed higher cross-reactivity of 20–32 percent with reciprocal formation of an insoluble complex. Typical invisible cross-reactions were demonstrated between proteases of different classes, namely two bromelains (*Monocotyledoneae*) and papain or ficin (*Dicotyledoneae*).

Studies of the localization of this antigenic determinant common to the four kinds of proteolytic enzymes and on the correlation between conformational and chemical homologies are now in progress.

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