Comparison between the Antigenic Structure of Mutually Related Enzymes. A Study with Papain and Chymopapain*

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ABSTRACT: Selective fractionation of antichymopapain antibodies according to inhibitory capacity was achieved by using a specific papain cross-immunoadsorbent. The antibodies which were adsorbed on the insolubilized papain are directed toward determinants common to papain and chymopapain, and are identical in their precipitability to antibodies selected by an analogous procedure from antipapain serum using a chymopapain immunoadsorbent. The antibodies to these "common" determinants inhibit much more efficiently the enzyme activity of papain and of chymopapain than antibodies specific exclusively to chymopapain. Comparison of the various antibody species obtained in the process of the selective fractionation and their relative reactivity with the two enzymes led to the conclusion that chymopapain contains more antigenic determinants than papain. Furthermore, at least one of these elicits the production of antibodies which inhibit papain activity not only on a macromolecular substrate but also on a small substrate. A schematic model for the distribution of antigenic determinants on papain and chymopapain is suggested.

In a previous communication (Arnon and Shapira, 1967) we have shown that antibodies toward papain may be selectively separated into two species which exhibit different inhibitory capacities. One of these antibody fractions was not inhibitory at all, with regard to the papain activity on a small molecular weight substrate, whereas the second fraction consisted of highly efficient inhibitors. This selective fractionation was accomplished by the use of an immunoadsorbent prepared from a cross-reacting enzyme–chymopapain. The inhibitory antibodies belonged to the species which had been adsorbed on this immunoadsorbent, namely, they were directed toward antigenic determinants common to papain and chymopapain; consequently they were capable of inactivating both enzymes. Recently, we demonstrated (Shapira and Arnon, 1968) that by employing a suitable immunization procedure, we could elicit the production of inhibitory antibodies against chymopapain as well. It was of interest, therefore, to examine whether selective fractionation of antichymopapain antibodies can be accomplished, similar to that obtained with antipapain antibodies. In other words, we wanted to determine whether antichymopapain antibodies could be fractionated by papain immunoadsorbent in the same way as the antipapain antibodies had been fractionated by chymopapain immunoadsorbent. If this were the case, the question could be raised as to what extent the antibodies specific to determinants common to both enzymes, but derived from the two different antisera, resemble each other, or whether they might even be identical.

We wish to report here the selective fractionation of antichymopapain antibodies and to describe the detailed properties of the various antibody species obtained. We also suggest a tentative model for the antigenic structure of papain and chymopapain, based on a comparison of the different antibody species derived from their respective antisera.

Materials and Methods

Materials

Papain (two-times crystallized lot 5623) and chymopapain (lot 5541) were purchased from Worthington Biochemical Co. Bz-Arg-Et[1] was obtained from Yeda Co. Casein was purchased from Nutritional Biochemical Co. Sephadex G-100 was from Pharmacia. All other reagents were either reagent grade or the best grade available.

Immunization Procedure. Rabbits were immunized by one injection of 10 mg of the respective enzyme in complete Freund's adjuvant (Difco), at multiple intradermal sites. This procedure was demonstrated (Shapira and Arnon, 1968) to be the method of choice for obtaining a high titer of antibodies, with uniformly high inhibitory

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Bz-Arg-Et, benzoyl-L-arginine ethyl ester; IgG, rabbit immunoglobulin G fraction; BrAc-cellulose, bromoacetylcellulose; Ch, total antibody population purified from antichymopapain IgG fraction by chymopapain immunoadsorbent; CCh, antibodies purified from antichymopapain IgG fraction with papain immunoadsorbent; ChS, chymopapain-specific antibodies adsorbed with chymopapain immunoadsorbent from the supernatant obtained after exhaustive adsorption of antichymopapain with papain immunoadsorbent; P, total antibody population purified from the antipapain IgG fraction by papain immunoadsorbent; CP, antibodies purified from antipapain IgG fraction with chymopapain immunoadsorbent; PS, papain-specific antibodies adsorbed with papain immunoadsorbent following exhaustive adsorption with chymopapain immunoadsorbent.

capacity. Starting 10 days after the injection, blood was collected weekly from the marginal ear vein. The antibody titer of the various individual sera, as determined by quantitative precipitin analysis, was 4–5 mg/ml, and remained at that level for several months without any booster injection.

Antibody Purification. Antibodies were purified from the IgG fractions of the antisera by the use of specific immunoadsorbents. The immunoadsorbents of both papain and chymopapain were prepared by binding the respective enzyme to Br-Ac-cellulose according to Robbins *et al.* (1967). One sample of the antibodies was purified from specific immune precipitate according to Givol *et al.* (1962), by dissolution in 0.2 M acetic acid and gel filtration on Sephadex G-100 column. Both methods of purification were described in detail in an earlier communication (Arnon and Shapira, 1967).

Quantitative Precipitin Test. Precipitin reactions were performed with whole antisera or purified antibody preparations. Increasing amounts of the antigen were added to a constant amount of the antibody. The precipitates formed after 1 hr at 37° and overnight at 4° were washed, dissolved in 0.1 M NaOH, and quantitated by measuring the absorbancy at 280 mμ within 10 min.

The amounts of antigen in the precipitates were determined in several cases after its labeling with ^{125}I (Amersham), according to the method of McFarlane (1958). The antibody content was then obtained from the measured absorbancy after deducting the calculated absorbancy of the antigen.

The amount of antigen bound to the antibody in the form of soluble antigen–antibody complexes was determined by adding goat antiserum against rabbit IgG to the supernatant of the precipitin tubes and monitoring the radioactivity in the precipitates.

Immunodiffusion. Double gel diffusion in agar gel was carried out in plates according to Ouchterlony (1948), at 22–25°.

Enzymatic Assays. The catalytic activity of the two enzymes was assayed in the presence of 0.005 M cysteine and 0.002 M EDTA. With the high molecular weight substrate, casein, the determination of the activity was analogous to the assay of trypsin (Kunitz, 1947), and was carried out as described in a previous publication (Arnon and Shapira, 1967). The reaction with papain was allowed to proceed for 10 min, and that with chymopapain for 60 min, before its termination by the addition of trichloroacetic acid solution. The activity on the low molecular weight substrate was assayed by following the hydrolysis of Bz-Arg-Et (0.08 M in 0.15 M NaCl) at pH 6.5 and 37°, using an autotitrator (Radiometer, Copenhagen, Model TTT1, with a combined electrode). In the case of papain 0.1 N NaOH was used for the titration, whereas in the case of chymopapain 0.01 N NaOH was used and the whole procedure was performed under an atmosphere of nitrogen.

Inhibition Assays. Experiments of inhibition were done only with purified antibody preparations. The extent of inhibition of the catalytic activity, on both high and low molecular weight substrates, was determined and calculated as described previously by Arnon and Shapira (1967). As described in that report, purified antibody preparations were devoid of any proteolytic activity, or nonspecific antiproteolytic activity.

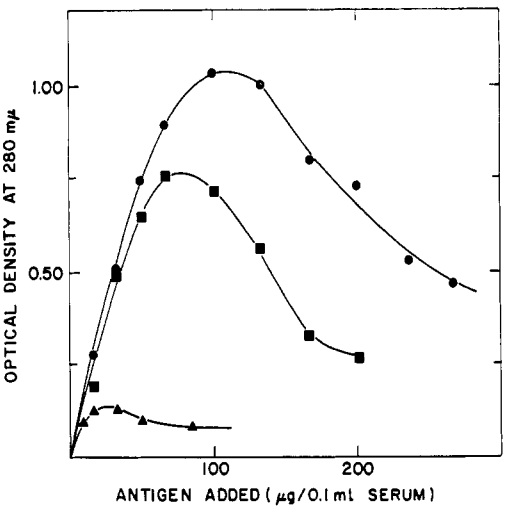

FIGURE 1: Precipitin curves of the following interactions: antichymopapain serum–chymopapain (●—●), antichymopapain serum–papain (▲—▲), and antichymopapain serum following absorption with papain in the equivalence zone–chymopapain (■—■).

The kinetic parameter, K_m, and the K_i values for the different antibody species, were calculated from the slopes of the respective Lineweaver and Burk plots according to Dixon and Webb (1964).

Physical Methods. Spectrophotometric measurements were made on a Zeiss Model PMQII spectrophotometer, at approximately 25°, with quartz cells of 1-cm light path. Radioactivity was measured in a well-type Tracerlab scintillation counter. Sedimentation measurements were carried out in a Spinco Model E ultracentrifuge, at 20–22° with the schlieren optical system. The samples were sedimented at 59,780 rpm.

Results

The quantitative precipitin reaction of chymopapain with its antiserum is shown in Figure 1. The same figure also illustrates the existence of a cross-reaction between the antichymopapain serum and papain. As shown, papain precipitates approximately 10% of the antibodies. Furthermore, after adsorption of the antiserum (by cross-precipitation with papain in the equivalence zone), the precipitin curve obtained with chymopapain was lower, implying the removal of a portion of the antibodies by papain. This finding provided the basis for the selective fractionation of the antibodies which was carried out in a manner analogous to that employed in the selective separation of antipapain antibodies (Arnon and Shapira, 1967). The IgG fraction obtained from antichymopapain serum was adsorbed with papain immunoadsorbent. The adsorbed antibodies, designated CCh, were eluted and the adsorbent was used for several consecutive adsorptions of the same IgG solution, until no more antibodies were eluted. The supernatant was then adsorbed with chymopapain immunoadsorbent and the antibodies, specific solely for chymopapain, and desig-

TABLE I: Properties of the Various Antibody Species Prepared from Antipapain Serum and Antichymopapain Serum.

Source of Antibodies	Antibodies	Description of Antibody Species	Inhibition in Antibody Excess				Immunol Reactivity	
			Papain		Chymopapain			
			Casein	Bz-Arg-Et	Casein	Bz-Arg-Et	Papain	Chymopapain
Antipapain serum	P	Total antipapain	92	65	52	43	+++	+
	CP	Common with chymopapain	93	97	92	77	++	++
	PS	Uniquely papain specific	74	7	7	4	+++	−
Antichymopapain serum	Ch	Total antichymopapain	37	40	93	60	+	+++
	CCh	Common with papain	92	94	92	77	++	++
	ChS	Uniquely chymopapain specific	6	3	60	44	−	+++

nated ChS, were eluted. In one preparation, selective separation of the antibodies was carried out by using the specific immune precipitates. In that case the anti-chymopapain serum was mixed with the amount of papain corresponding to the zone of maximum precipitation. The precipitate was dissociated with acid and the antigen was removed by gel filtration to yield the CCh species of antibodies. The ChS species was obtained analogously from the resultant supernatant by precipitating the antibodies with chymopapain. The ChS species obtained by this procedure were not completely identical to those obtained on the immunoadsorbent since they were still contaminated with antibodies of the CCh type, which had not been quantitatively precipitated. On the other hand, the CCh antibody species obtained in this way proved to be similar in every respect to the antibodies obtained using the immunoadsorbents.

The two species of antibodies, namely CCh and ChS, and the total antichymopapain antibody population (obtained from the IgG fraction of the antiserum by direct adsorption with chymopapain immunoadsorbent, and designated Ch) were interacted with both chymopapain and papain in agar gel (Figure 2). It can be seen that chymopapain reacts with all three species of antibody, while papain, on the other hand, precipitates with Ch and CCh only, and does not interact at all with the ChS antibodies. This is exactly parallel to the interaction of the two enzymes with the three species of antibodies obtained from antipapain serum and described previously (Arnon and Shapira, 1967). The spur at the intersection of the precipitin bands of chymopapain and papain with the total antibody population (Ch) provides further proof that papain interacts with only a fraction of the antibodies, and that this fraction is identical with the CCh species defined above. The CCh antibodies gave a band of identity with both enzymes.

The various species of antibodies obtained from the antisera toward both chymopapain and papain, and their significant characteristics, are listed and summarized in Table I. Besides the unfractionated antibody preparations, only two out of the various selected species of antibodies are immunologically reactive with both papain and chymopapain, and these two are directed toward the common determinants shared by the two enzymes. Their interaction with each of the two enzymes was quantitated by the precipitin test (Figure 3). It can be seen that each enzyme reacts identically with the two antibody species. However, chymopapain is shown to be a better precipitating agent than papain with either species of antibodies, both the one derived from its homologous antiserum and the one derived from antipapain serum. This suggests that the observed differences in the various precipitin curves are due not to dissimilarity in the antibodies, but rather to variation in precipitating reactivity of the two enzymes.

In order to elucidate this point, the precipitin experiment was repeated with labeled antigens, and the soluble antigen–antibody complexes were further precipitated by adding to the supernatant fluids goat antiserum toward rabbit IgG. By monitoring the radioactivity and combining the calculated amounts of the enzyme present in both the direct enzyme–antienzyme precipitates

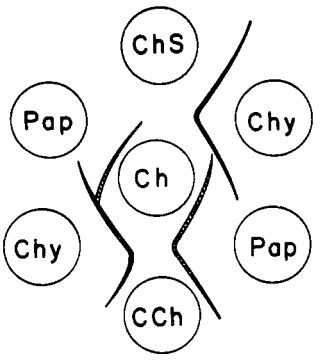

FIGURE 2: Double diffusion in agar gel. Ch, CCh, and ChS are the antibody species. Pap, papain; Chy, chymopapain.

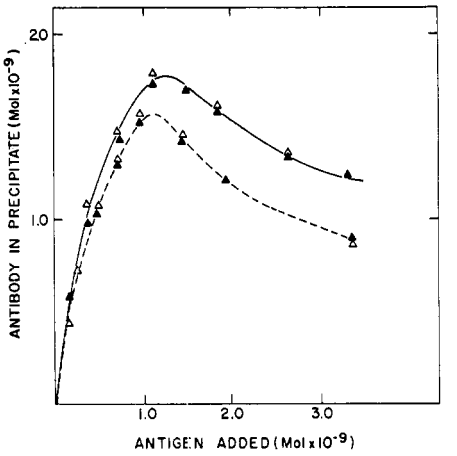

FIGURE 3: Precipitin curves of chymopapain (—) and papain (- - - - -) with the CCh antibody species (▲) and CP antibody species (△). Antibody (2.5×10^{-9} M) was used in each precipitin tube.

and the indirect precipitates with the goat antibodies, it was found that the total molar amounts of the two enzymes which were bound by each of the antibody species were the same (Figure 4). This indicates that the variations observed previously in the extent of the precipitin reactions were indeed due to differences in the relative precipitability of papain and chymopapain, rather than to their capacity to combine with each of the two antibody species. The significance of this conclusion with respect to the location of the antigenic determinant on the two enzymes will be discussed later.

The inhibitory effect of all the antibody types mentioned in Table I, on both chymopapain and papain, was measured both with a high molecular weight substrate and with a low molecular weight substrate. Figure 5 summarizes the results obtained with casein as the substrate. The left side of the picture shows the inhibitory effects on each of the two enzymes (A, papain; B, chymopapain) by the antibody species derived from the respective homologous antisera, whereas the right side (C and D) includes the results of the inhibition experiments carried out on each of the two enzymes with the antibody species derived from the respective heterologous antiserum. It is seen that each enzyme is inhibited by its homologous total antibody populations as effectively as by the fraction common to the two enzymes, whereas the fraction uniquely specific for each enzyme exhibits a lower inhibitory effect. These findings can be readily accounted for when it is borne in mind that with such a large substrate the steric effect of the antibodies is quite large, and that, therefore, most of the antibodies are capable of interfering with the catalytic activity of the homologous enzyme. On the other hand, it is clear from Figure 5C,D that the antibodies specific exclusively to one enzyme do not inhibit the other enzyme at all. Thus, the PS species do not inhibit at all chymopapain activity and likewise the ChS species do not inhibit papain. As far as the inhibition of enzymatic hydrolysis of casein is concerned, the selectivity of the fractionation of the antibodies is thus accentuated when the inhibitory effect is measured on the heterologous enzyme. This is due to the fact that the antibodies specific to antigenic determinants present only on one enzyme do not combine at all with the other enzyme, and consequently do not exhibit any inhibitory effect.

The inhibitory effects of the selectively separated antibodies on the catalytic activity on Bz-Arg-Et are depicted in Figure 6. In inhibiting papain (Figure 6A), the CP and CCh antibody species did not exhibit an identical effect. Ultimately, namely in antibody excess, they did bring about the same extent of inhibition, but in lower concentrations the CP species was a more efficient inhibitor. This phenomenon was further emphasized in the attempt to measure the kinetics of inhibition of papain by the two antibody species (Figure 7). As can be seen, the K_i value obtained with the CCh species (10.3×10^{-6}) is approximately twice as large as that obtained with the CP species (4.6×10^{-6}). This difference is in

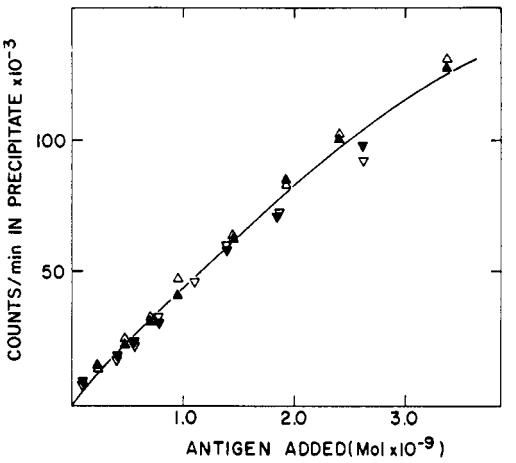

FIGURE 4: Antigen binding capacity of the antibody species common for the two enzymes. The experiment was carried out by adding increasing amounts of labeled (^{125}I) papain and chymopapain (specific radioactivity 50×10^3 cpm per 10^{-9} M) to each of the antibody preparations (2.5×10^{-9} M of antibody per tube), and subsequent precipitation of the soluble antigen–antibody complexes in the supernatant fluids with goat anti-(rabbit IgG) serum. The values in the figure represent the combined radioactivity present in both the direct enzyme–antienzyme precipitates and the indirect precipitates with the goat antibodies. (▲) CCh antibodies with papain; (△) CP antibodies with papain; (▼) CCh antibodies with chymopapain; (▽) CP antibodies with chymopapain.

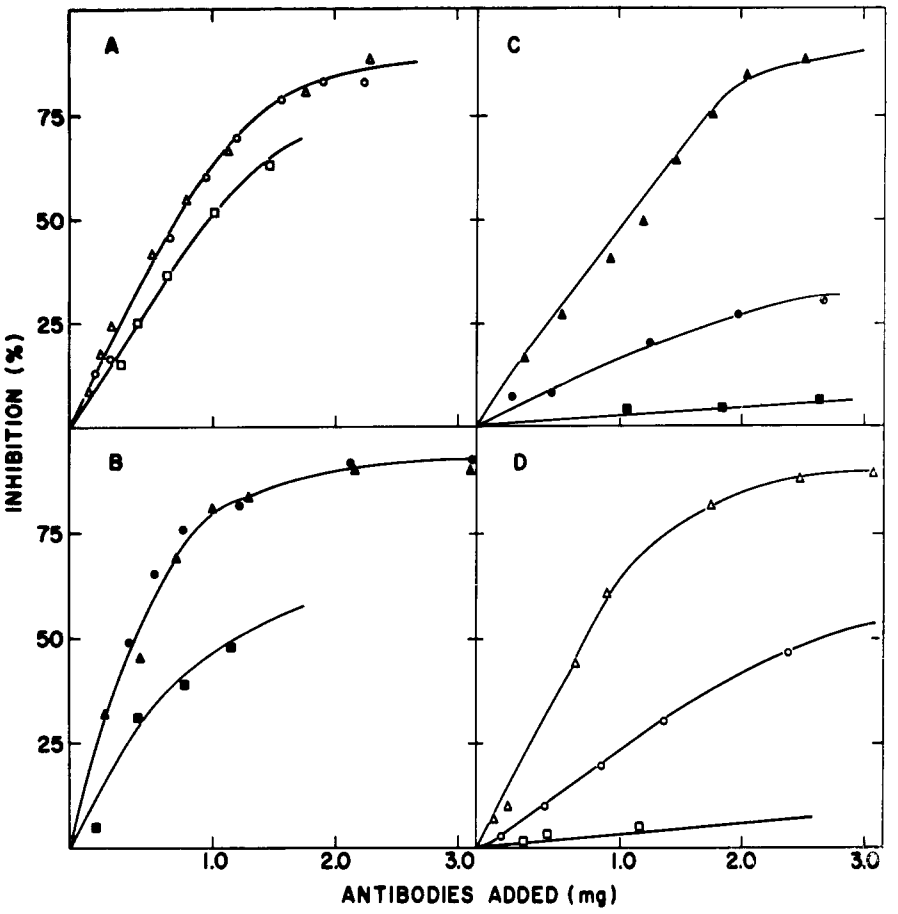

FIGURE 5: Inhibition of papain and chymopapain (2.4×10^{-9} M) activity on casein as substrate by the various antibody species. (A) Inhibition of papain by its homologous antibody species P (○), CP (△), and PS (□). (B) Inhibition of chymopapain by its homologous antibody species Ch (●), CCh (▲), and ChS (■). (C) Inhibition of papain by the heterologous antibody species Ch, CCh, and ChS. (D) Inhibition of chymopapain by the heterologous antibody species P, CP, and PS.

agreement with the difference in the inhibition effect by the two antibody species at the concentrations at which the K_i values were determined.

Experiments with chymopapain (Figure 6B) demonstrate that the two antibody species common to the two enzymes, namely CP and CCh, inhibit to the same extent. On the other hand, the ChS antibodies, namely those directed toward determinants that are not present on papain, are capable of inhibiting chymopapain even on a small substrate. This is in contrast to the finding that the counter-analog PS species is not inhibitory at all on its homologous enzyme, papain. This finding leads to the conclusion that one of the determinants of chymopapain which is not present on papain elicits the formation of antibodies, inhibitory to both large and small molecular weight substrates. It should be noted that both CP and CCh species inhibit the activity of papain almost entirely, but their inhibition of chymopapain, even in large antibody excess, does not exceed 77%.

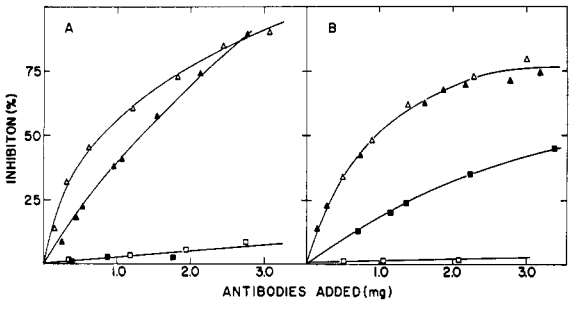

FIGURE 6: Inhibition of papain (A) and chymopapain (B) activity of Bz-Arg-Et by the various antibody species. CP (△), CCh (▲), PS (□), and ChS (■). Each enzyme (2.4×10^{-9} M) was used for the assays. The total antibody population (P and Ch, respectively) yielded inhibition values intermediate between those observed with the two selectively separated species (Arnon and Shapira, 1967).

Discussion

This study demonstrates that antibodies to chymopapain can be selectively separated into two populations that differ in their inhibitory capacity, in analogy to the selection effected on antipapain antibodies reported in an earlier publication (Arnon and Shapira, 1967). This selection was achieved by cross-adsorption of the antiserum to each of these two enzymes with the heterologous enzyme. In this manner two species of antibodies were obtained, designated CP and CCh, which could be expected to be identical, since each contained only those antibodies which were directed toward antigenic determinants present on both enzymes.

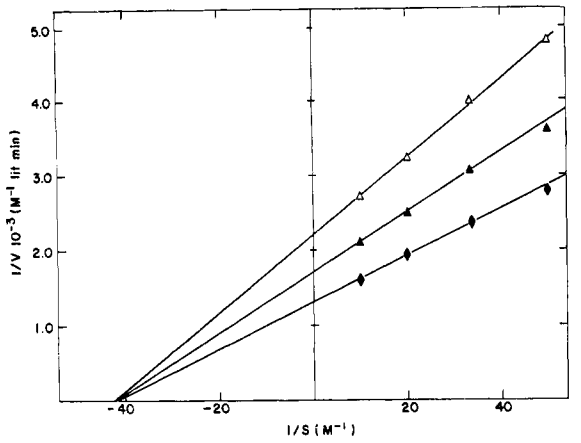

FIGURE 7: Analysis of the inhibition of papain (2.4×10^{-9} M) by CP (△—△) and CCh (▲—▲) antibody species (0.5 mg). (◆—◆) No antibodies added.

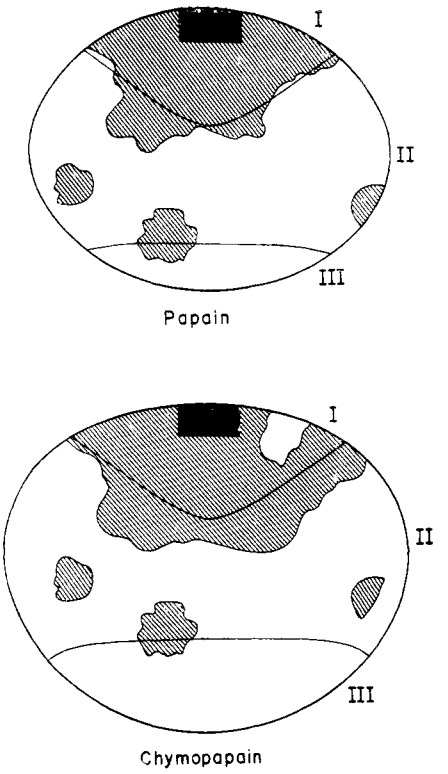

FIGURE 8: Schematic model of antigenic structure of papain and chymopapain. The black area represents the active site of each enzyme. The shaded areas represent the determinants which are common for the two enzymes, whereas the remaining area represents the uniquely specific determinants. Antibodies to zone I are inhibitory for both low and high molecular weight substrates, antibodies directed toward zone II are inhibitory only for large substrate, whereas antibodies to zone III are noninhibitory.

The most intriguing point of this study was to find out whether these two species, though elicited by different antigens, were actually identical. Indeed, in their immunological interaction with either enzyme, the two common antibody species showed complete identity, both in quantitative precipitin (Figure 3) and in their capacity to bind the antigens (Figure 4). Similarly, they exhibited an identical inhibitory capacity toward each of the two enzymes regarding the catalytic activity on a large substrate (Figure 5). Moreover, they affected the catalytic activity of chymopapain on a low molecular weight substrate to the same degree (Figure 6B). However, a dissimilarity between the two antibody species is noted in the inhibitory effect on papain with respect to its activity on a low molecular weight substrate (Figure 6A), although this difference does not persist in high antibody concentration. In the antibody concentration region where differences were observed in the extent of inhibition, the K_i values of the two species were also different. This phenomenon might be due to a difference in the affinities of the two species, due to the same antigenic determinants being present on different carriers. Such effects of the carriers on the affinities of the elicited antibodies have been reported for other cases and discussed by Crumpton and Wilkinson (1965). This difference between the two antibody species was unnoticed with chymopapain probably because of the relatively low activity of this enzyme on Bz-Arg-Et and the low affinity of substrate to enzyme.

Even though papain and chymopapain, being derived from the same source, papaya latex, are very similar in their enzymic properties and their substrate spectrum (Ebata and Yosunobu, 1962), the percentage of antibodies which are common to the two enzymes is small. Cross-precipitation shows the presence of only 10% common antibodies in antichymopapain serum, but a larger fraction which, on the average, amounted to 20% in antipapain serum. This finding corroborates the conclusion that antichymopapain serum contains relatively more antibodies to antigenic determinants that are not present on papain.

The chymopapain molecule is larger than the papain molecule; according to Ebata and Yosunobu (1962), its molecular weight is 27,000, whereas that of papain is 20,700 (Kimmel and Smith, 1954). It could therefore be expected in all likelihood to contain a larger number of antigenic determinants. The results of this study offer three independent conclusions which are relevant to this assumption. (a) Chymopapain contains at least one determinant, not present on papain, which elicits antibodies that are inhibitory for both large and small substrates, whereas papain does not contain an equivalent determinant. This is deduced from the inhibitory capacity of ChS antibodies on chymopapain (Figure 6B), in contrast to the complete lack of inhibitory activity in PS antibodies (Figure 6A,B). (b) The antibodies which are common to the two enzymes, namely the CP and CCh species, form more soluble complexes with papain than with chymopapain (yielding lower precipitin curves (Figure 3) but having identical antigen binding capacity (Figure 4). Likewise, their inhibitory effect on papain activity on a small substrate is greater than the effect on chymopapain activity, even in antibody excess (Figure 6). These phenomena may be due to the fact that the respective common antigenic determinants are closer to each other and to the catalytic site in the papain molecule than in the chymopapain molecule. (c) In the inhibition experiments with casein as the substrate (Figure

5) the total antibody population is as inhibitory on the homologous enzyme as the antibody fraction which is common to the two enzymes, whereas the specific antibody species are less effective inhibitors. This fact indicates that even though most of the antibodies can inhibit the catalytic activity of the homologous enzyme on a large substrate, nevertheless a small portion of noninhibitory antibodies does exist, and these belong mainly to the species which is specific for each homologous enzyme only, and hence less inhibitory. This phenomenon is more clearly pronounced with chymopapain.

On the basis of all the information accumulated here a schematic model is suggested for the distribution of the antigenic determinants on papain and chymopapain (Figure 8). According to Drenth *et al.* (1967) the papain molecule is spheroidal, and its dimensions are 36 × 48 × 36 Å. The chymopapain molecule is larger than the papain molecule. The active area of each enzyme is represented in the figure by the black site. Regions or determinants shared by the two enzymes are shaded, whereas the light area is distinctly specific for each enzyme. Since we have proved previously (Shapira and Arnon, 1967) that the mechanism of inhibition by the antibodies is *via* steric hindrance, each enzyme can be looked upon as consisting of three zones. Antibodies to determinants in zone I, due to the proximity of their interaction loci to the active site, will inhibit the catalytic activity even on a low molecular weight substrate. Antibodies interacting with determinants in zone II will inhibit the enzyme action only on a large substrate, whereas antibodies to determinants in zone III are completely noninhibitory. The similarity between the two enzymes is located mainly in zone I, although zone II also contains regions or determinants which are shared by the two enzymes. Zone III contains mainly determinants which are unique for each enzyme, and elicit the formation of antibodies which will not interact at all with the heterologous enzyme. However, as pointed out in the model, at least one such unique determinant of chymopapain is located in zone I and elicits antibodies which are effective inhibitors, but specifically and only to the homologous enzyme. There is evidence that an equivalent determinant is not present in papain.

It was recently reported (Atassi and Saplin, 1968) that in sperm whale myoglobin the antigenic determinants are within regions which occupy mostly the corners in the three-dimensional structure of the molecule. If this is a general phenomenon, applicable for other proteins as well, the knowledge of the primary amino acid sequence, and the three-dimensional structure of the molecule, may be helpful in the location of the antigenic determinants on the molecule. A study of the type described in the present article, namely, the comparison of the antigenic structure of two related enzymes, can shed light on the correlation between the structure and the various biological activities of the molecule.

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