Antibodies to Papain. A Selective Fractionation According to Inhibitory Capacity*

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ABSTRACT: Selective separation of antibodies to papain, with different inhibitory properties, was attempted by applying a series of methods for their purification. The various antibody preparations were characterized and compared on the basis of their capacity to inhibit the enzymatic activity of papain, and of their relative reactivity in the precipitin reaction. The antibodies were isolated either by specific precipitation with the homologous antigen (or with mercuripapain), followed by acid dissociation and removal of the antigen by gel filtration, or by adsorption on a specific insoluble immunoadsorbent followed by elution. All the antibody preparations, similarly to the antibodies in the whole immunoglobulin G fraction, inhibited papain activity on benzoyl-L-arginine ethyl ester to the same extent, namely 55–60%. Selection of antibodies differing in their inactivating capacity has been achieved by using an insoluble immunoadsorbent prepared with the cross-reacting enzyme chymopapain. Antibodies isolated thereby inhibited papain activity to the extent of 92%. On the other hand, the antibodies remaining in solution following exhaustive cross-adsorption by chymopapain immunoadsorbent, did not inhibit papain activity at all although they precipitated well with it. It is concluded that the antibody population is heterogeneous, and is constituted of distinct species of antibodies, the formation of which was triggered by different antigenic specificity determinants on the enzyme.

The observed enzymic activity of papain in its immunospecific precipitates reflects the probability of its interaction with each of these different species.

The properties of the immunological system papain–antipapain have been described in a previous communication (Arnon, 1965). One of the manifestations of the interaction of papain with its antiserum was reported to be partial inhibition of the catalytic activity of the enzyme. This phenomenon is not unique for papain. A survey of the literature on the interaction of various enzymes with their respective antisera shows that most enzymes are partially or completely inhibited by their antibodies (reviewed by Cinader, 1963). The mechanism of this inhibition, however, is not clearly understood and conceivably may differ from one enzyme to another, depending on their structural features and mode of action. Moreover, the inhibitory capacity is related to the size of the sustrate as well —the higher its molecular weight, the more effective the inhibition. Another factor which may determine the type of inhibition and the capacity of various anti-enzyme antisera to inhibit enzymic activity is the heterogeneity of the antibodies elicited by each enzyme.

One of the features characterizing the inhibition of many enzymes by their respective antibodies is the residual catalytic activity persisting even in extreme antibody excess, an activity which is not reduced by the addition of more antibody (*e.g.*, Gregory and Wróblewski, 1958; Ultmann and Feigelson, 1963). This phenomenon can be interpreted in two ways. One possible explanation is that the antibodies inhibit according to a uniform mechanism; each enzyme molecule is partially inhibited, while retaining a residual enzymic activity after its combination with the antibody. Alternatively, the antibody population could be regarded as being inherently heterogeneous, consisting of species which differ in their inhibitory capacity. In the latter case, fractionation of the antibodies could presumably be used to separate such species and to isolate those antibodies which combine more specifically with antigenic determinants associated with the catalytic active site of the enzyme. The presence of nonneutralizing antibodies in an antienzyme antiserum has been demonstrated for the system lysozyme–anti-lysozyme (Shinka *et al.*, 1962). Moreover, there have been indications that fractions of differing inhibitory capacity could be obtained from a single pool of antibodies. Thus, Cinader and Lafferty (1964) have demonstrated that the Fab univalent antibody fragments, I and II (Porter, 1959), prepared from antiribonuclease immunoglobulin differ in their inhibitory potency, fragment I possessing five times as much neutralizing activity as fragment II. Fractionation was thus achieved, in this case, on the basis of the electrical charge of the antibody fragments. An equivalent type of separation performed on the system papain–antipapain yielded contradictory results (Shapira and Arnon, 1967a). The fractionation of purified antipapain antibodies on DEAE Sephadex yielded two species with identical inhibitory capacity. Thus it appears that in the system

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papain–antipapain, at least, differences in the net charge of the antibodies are not reflected in their inhibitory properties.

The problem of selective separation of antibodies may be tackled from a different angle. It may be assumed that cross-inhibition in enzyme–antienzyme systems, *i.e.*, inhibition of a particular enzyme by antibodies to another enzyme, can be taken as an indication that the catalytic centers or related regions in the two enzymes bear some similarity to each other. This assumption is corroborated by the finding that antitrypsin antibodies indeed do inhibit chymotrypsin, but not subtilisin (Arnon and Schechter, 1966). Thus, an enzyme with catalytic properties similar to those of papain could be expected to cross-react and to be cross-inhibited with a fraction of the antipapain antibodies, and, therefore, might distinguish between different species of antibodies.

We wish to report here the successful fractionation of antibodies against papain, according to inhibitory capacity, making use of specific immunoadsorption with insolubilized chymopapain. Using this procedure, efficient inhibitory antibodies have been separated from a species almost completely devoid of inhibitory properties.

Materials and Methods

Materials. Papain (two-times crystallized, lots 5581, 5623), mercuripapain (crystallized, lot 6116), and chymopapain (lot 5541) were purchased from Worthington Biochemical Co. Benzoyl-L-arginine ethyl ester (BAEE)[1] was obtained from Yeda Co. Casein was purchased from Nutritional Biochemical Co. Sephadex G-100 and G-150 were from Pharmacia. DEAE-cellulose and CM-cellulose were from Bio-Rad Laboratory. All other reagents were either reagent grade or the best grade available.

Enzymatic Assays. Papain activity 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 in the following manner. Casein solution (1 ml of 1%) (in 0.15 M NaCl–0.05 M Tris buffer, pH 8.0, containing cysteine and EDTA) was added to 1 ml of papain solution containing 5–50 μg of papain. Following 10-min incubation at 37°, 4 ml of a 5% solution of trichloroacetic acid was added. After 1 hr the precipitates were filtered and the absorbancy of the solution at 280 mμ was determined. Since the activity is not linear in this range of concentrations, the activity of unknown solutions was expressed as micrograms of enzyme, according to the calibration curve. The activity of papain on low molecular weight substrate was assayed by following the hydrolysis of BAEE (0.08 M in 0.15 M NaCl) at pH 6.5 and 37°, with 0.1 N NaOH, using an autotitrator (Radiometer, Copenhagen, Model TTT 1, with a combined electrode). The activity of chymopapain on BAEE was determined in a similar manner, under an atmosphere of nitrogen, using 0.01 N NaOH.

Inhibition Assays. Inhibition of the activity of the enzyme on casein was assayed by adding increasing amounts of the particular antiserum, immunoglobulin fraction or purified antibody preparation, to 25 μg of papain. The volume was brought to 1 ml with 0.15 M NaCl–0.05 M Tris buffer at pH 8.0. Following incubation for 1 hr at 37°, the activity was determined in the usual manner. The extent of inhibition was calculated from the residual activity as given in eq 1

$$\text{inhibition} = 100 \times \frac{\text{activity of uninhibited enzyme} - \text{activity of inhibited enzyme}}{\text{activity of uninhibited enzyme}} \quad (1)$$

Whole sera (of normal rabbits) were found to cause nonspecific inhibition of papain activity, in a manner similar to that known to occur with horse sera (Pochon, 1944). This effect was not observed with the immunoglobulin fraction of the sera, but persisted in the globulin-free serum. The results of all the inhibition experiments carried out with antisera were, therefore, corrected by substraction of the corresponding inhibition caused by the same sample of serum freed of its globulins.

Inhibition of the hydrolysis of BAEE catalyzed by papain and chymopapain was carried out with the IgG fraction or with purified antibody preparations. The activity was assayed following incubation of the enzyme with the particular inhibitor for 1 hr at 37°, in a total volume of 1 ml of 0.15 M NaCl. The extent of inhibition was calculated from the initial rates of the reaction in the absence and in the presence of increasing amounts of inhibitor: inhibition = $100-(v_0 - v)/v_0$.

Immunization Procedure. Rabbits were immunized by one injection of 10 mg of papain in complete Freund's adjuvant (Difco), at multiple intradermal sites. Starting 10 days after the injection, blood was collected weekly from the marginal ear vein. The antibody content as determined by quantitative precipitin analysis was at least 3 mg/ml, and remained at that level for several months without any booster.

Preparation of Immunoglobulin G (IgG) Fraction. The IgG fraction was isolated from either the antiserum or normal rabbit serum by four to five successive precipitations with ammonium sulfate. The first precipitation was carried out at 40% saturation while

[1] Abbreviations used: DEAE, diethylaminoethyl; BAEE, benzoyl-L-arginine ethyl ester; IgG, rabbit immunoglobulin G fraction; BAC, bromoacetylcellulose; CP, antibodies purified from antipapain IgG fraction with chymopapain immunoadsorbent; PS, antibodies adsorbed with papain immunoadsorbent following exhaustive absorption with chymopapain immunoadsorbent; P total antibody population purified from the antipapain IgG fraction by papain immunoadsorbent.

all the consecutive ones at 33% saturation. Saturated ammonium sulfate solution, adjusted to pH 7.0, was prepared at 4°. The dissolved precipitate was dialyzed exhaustively against 0.15 M NaCl.

Since IgG preparations of normal rabbit serum may contain proteolytic activity (Robert and Beckman, 1967), several procedures for the preparation of IgG were tested. The procedure described above was the method of choice for obtaining IgG preparation devoid of proteolytic activity (Table I). The immunoglobulin

TABLE I: Proteolytic Activity in Preparations of Normal Rabbit IgG Obtained by Different Procedures.[a]

Prepn	Procedure	Act. ($M\ min^{-1} \times 10^{-3}$)
1	Precipitation with $(NH_4)_2SO_4$ (40% saturation) and chromatography on DEAE-cellulose in 0.0175 M phosphate buffer (pH 6.3)[b]	2.18
2	Rechromatography of preparation 1 under the same conditions	1.24
3	Rechromatography of preparation 1 on Sephadex G-100 in 0.5 M NaCl–0.02 M Tris buffer (pH 8.0)	1.64
4	Rechromatography of preparation 1 on CM-cellulose in 0.01 M sodium acetate buffer (pH 5.5), with gradient of 0.9 M acetate buffer (pH 5.5)	1.65
5	Four successive precipitations with $(NH_4)_2SO_4$ (33% saturation)	0.014
6	Purified antibody preparation.	<0.004

[a] Activity of 10 mg of IgG preparation was assayed on BAEE (0.08 M). [b] Levy and Sober (1960).

solutions were stored in the frozen state. The protein concentration was determined spectrophotometrically, assuming a specific extinction coefficient of $E^{1\%}_{1\ cm}$ 14.0 at 280 mμ (Porter, 1959).

Antibody Purification. (1) From immune precipitates, according to Givol *et al.* (1962): Specific antibodies were precipitated from the antiserum either with papain or with mercuripapain in the equivalence zone. The washed precipitate (obtained from 100 ml of serum and 30 mg of papain or mercuripapain) was dissolved in 8 ml of 0.2 M acetic acid. The supernatant (containing about 370 mg of antibodies) was applied to a Sephadex G-100 or G-150 column (3.5 × 100 cm) previously equilibrated with 0.1 M acetic acid. Elution was carried out at 4° with the same solvent and the absorbancy of the effluent solution was measured at 280 mμ. The fractions under the main peak were pooled, dialyzed exhaustively against 0.15 M NaCl, and concentrated by vacuum dialysis.

(2) With specific papain immunoadsorbent: The immunoadsorbent was prepared by binding papain to bromoacetylcellulose (BAC) (Jagendorf *et al.*, 1963), according to Robbins *et al.* (1967), with the following modifications. The binding of the papain was carried out by dispersing BAC (an amount equivalent to 1 g of dry powder, containing 8.5% bromine) in a solution of 400 mg of papain in 0.05 M citrate buffer (pH 3.8) containing 0.005 M cysteine and 0.002 M EDTA (50 ml). The treatment with 8 M urea was omitted since it effects papain activity. This treatment is not necessary in this case as no significant amount of noncovalently bound papain was eluted with 8 M urea. In different immunoadsorbent preparations the protein concentration ranged from 1.75 to 3.25% (nitrogen analysis, Dumas). Under these binding conditions (low pH) most of the binding occurs *via* the sulfhydryl groups of the protein (Jagendorf *et al.*, 1963). The resulting immunoadsorbent did not show any detectable proteolytic activity on either BAEE or casein (in amounts containing up to 10 mg of enzyme).

The antibodies were adsorbed from antisera or IgG preparations and eluted with 0.1 M acetic acid as described by Robbins *et al.* (1967). Following exhaustive dialysis against 0.15 M NaCl they were concentrated by vacuum dialysis. They sedimented in the ultracentrifuge as a homogeneous peak with $s_{w,20} = 6.5$ S, and did not exhibit any residual proteolytic activity (Table I).

In the same manner antibodies were adsorbed on and eluted from a chymopapain immunoadsorbent. The chymopapain immunoadsorbent was prepared similarly to the papain immunoadsorbent, by dispersing BAC (an amount equivalent to 1 g of dry powder with a bromine content of 8.2%) in a solution of 350 mg of chymopapain in 0.05 M citrate buffer (pH 3.8) containing 0.005 M cysteine and 0.002 M EDTA (50 ml). The final product contained 3.0% protein.

Quantitative Precipitin Test. Precipitin reactions were performed with whole antisera, IgG fractions, 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 was determined in several cases after its labeling with ^{131}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, assuming the molar extinction of papain at 280 mμ to be 5.1×10^4 (Glazer and Smith, 1961).

Immunodiffusion. Double diffusion in agar gel was carried out in plates according to Ouchterlony (1948).

Physical Methods. Spectrophotometric measurements were made on a Zeiss Model PMQII spectrophotometer,

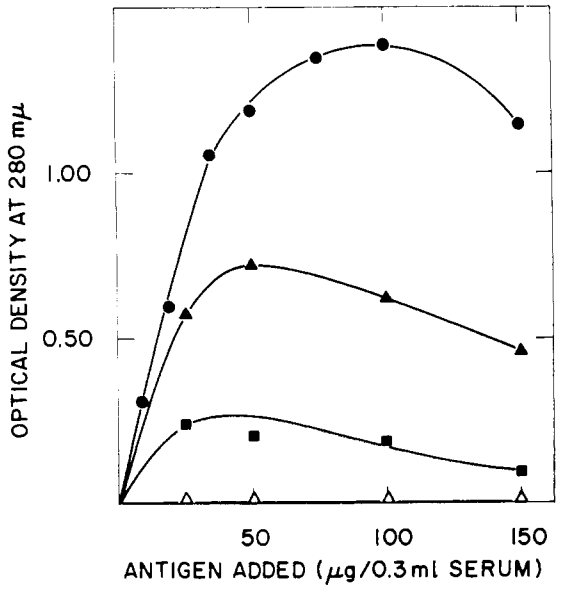

FIGURE 1: Precipitin curves of papain with: whole antiserum (●—●), supernatant after first immunoadsorption (▲—▲), supernatant after second immunoadsorption (■——■), and supernatant after third immunoadsorption (△——△).

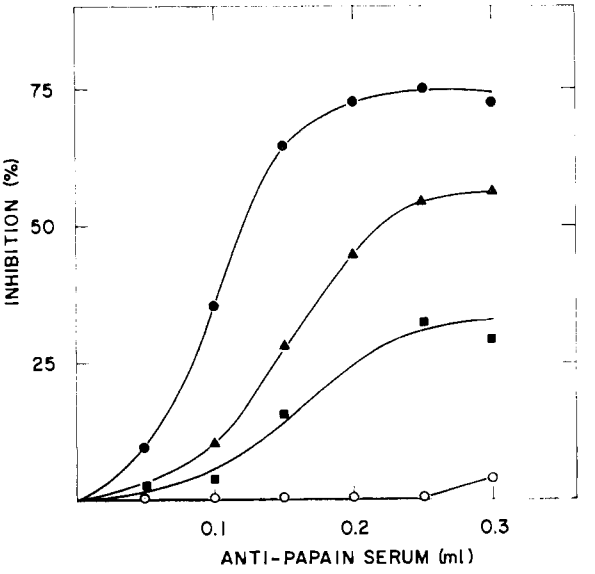

FIGURE 2: Inhibition of papain activity on casein by: whole antiserum (●——●), supernatant after first immunoadsorption (▲——▲), supernatant after second immunoadsorption (■——■), and supernatant after third immunoadsorption (O——O).

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

Several methods for isolation of antibodies were used in this study to achieve selective fractionation of antipapain antibodies. The various antibody preparations were characterized and compared, on the basis of their capacity to inhibit the enzymatic activity of papain and of their relative reactivity in the precipitin reaction.

Antibodies Isolated with Specific Immunoadsorbent. The immunoadsorbent used for the adsorption of antipapain antibodies was a preparation made by linking papain through its reactive sulfhydryl groups to the BAC carrier. It was first tested whether such an immunoadsorbent would selectively remove from solution those antibodies which would not affect the active site of the enzyme.

For this purpose, 150 ml of antiserum (containing 3 mg of antibody/ml) was subjected to three consecutive adsorptions with 2 g of the immunoadsorbent. The antibodies were eluted after each adsorption. The resultant purified antibody preparations were kept for subsequent detailed characterization, while samples of each supernatant fluid were tested for their precipitin and inhibitory activity. As Figure 1 indicates, each consecutive adsorption resulted in a decrease in the content of the precipitating antibody in the antiserum. This finding was parallelled by the observed decrease in the capacity of the serum samples to inhibit the enzymic activity of papain on casein (Figure 2). All the values used in Figure 2 to denote the extent of inhibition were calculated from the experimental data after introduction of the appropriate correction for nonspecific inhibition caused by the serum. As may be seen in Figures 1 and 2, the residual supernatant fluid obtained after three adsorptions was entirely devoid of both precipitating antibodies and inhibitory activity on papain.

An identical procedure was used to isolate the antibodies from the IgG fraction of the antiserum, with similar results. As with the whole antiserum, the adsorption of antibodies resulted in a corresponding decrease in precipitin activity and inhibitory capacity in the supernatant.

Antibodies Isolated from Immune Precipitates. A typical elution pattern of an acidic solution of a papain–antipapain precipitate from a G-100 Sephadex column is shown in Figure 3. The first, small peak, which had a very low level of papain activity (*ca.* 1 μg/ml of effluent) was found to contain aggregates of sedimentation constant 9.4 and 15 S. The material corresponding to the main peak (shaded zone) possessed no detectable papain activity, and migrated in the ultracentrifuge as a single sharp peak of $s_{w,20} = 6.5$ S. The third fraction consisted of papain, since it was found to account for most of the enzymatic activity (81 μg/ml) in the complex, and was devoid of any fraction that would migrate in the ultracentrifuge like IgG.

TABLE II: Analysis of the Precipitates Obtained in the Equivalence Zone between Papain and Various Preparations of Purified Antibodies.

Antibody Preparation	Extinction at 280-mμ Soln of Ppt	Papain in Ppt (μg)	Antibody in Ppt (μg)	Antibody: Antigen Ratio (*M*/*M*)
Unfractionated antipapain serum	2.10	80	1360	2.51
IgG fraction of antiserum	1.66	64	1072	2.48
AI[a]	0.86	32	562	2.60
AII	0.84	32	543	2.50
AIII	0.42	16	271	2.50
PI[b]	0.56	24	361	2.23
PII	0.55	24	355	2.19
ISP[c]	0.94	40	602	2.23
ISH	0.30	12	193	2.36

[a] A_I, A_{II}, and A_{III} antibodies purified from unfractionated antiserum by the first, the second, and the third adsorption, respectively, with specific papain immunoadsorbent. [b] P_I and P_{II}, antibodies purified from the IgG fraction of the antiserum by the first and second adsorption, respectively, with specific papain immunoadsorbent. [c] ISP and ISH, antibodies purified from specific immune precipitates of the antiserum with papain and mercuripapain, respectively.

In identical fashion, purified antibodies were isolated from an acidic solution of the precipitate obtained by reacting mercuripapain with antipapain serum. The results obtained were very similar, except for the fact that the first peak, which in the case of the papain–antipapain complex had consisted of the fraction containing aggregates, was now missing.

Precipitating Properties of the Various Antibody Preparations. The precipitability of papain with the various antibody preparations was studied by both gel diffusion and quantitative precipitin analysis. The various antibody preparations tested are listed in Table II. All were found to react with papain in the gel diffusion experiment to yield one merging precipitin band devoid of spur formation. This similarity between the various antibody preparations was underlined further by the results of the quantitative precipitin analyses; as shown in Table II, the composition of the precipitates obtained in the zone of equivalence was the same for all the preparations tested.

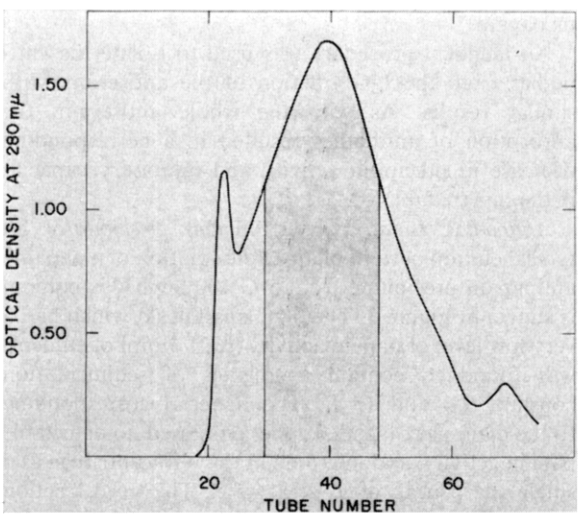

FIGURE 3: Elution pattern of the acid-dissolved papain–antipapain precipitate on Sephadex G-100 in 0.1 M acetic acid.

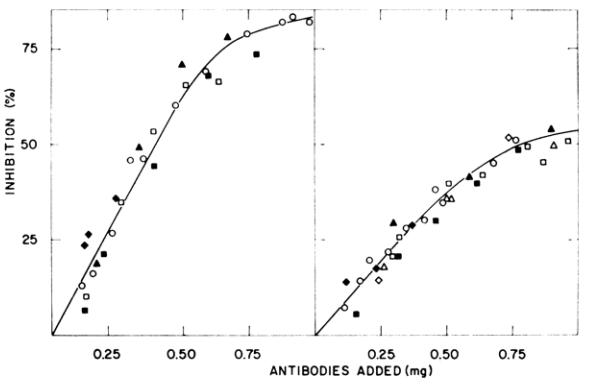

FIGURE 4: Inhibition of papain (25 μg/ml) activity by the various antibody preparations. Left side: Activity assayed on casein. Right side: Activity measured on BAEE. Since samples of purified antibodies are compared with the total IgG fraction (O—O), the amount of antibodies added was based on precipitin analysis. (■—■) AI, (▲—▲) AII, (◆—◆) AIII, (□—□) PI, (△—△) ISH, and (◇—◇) ISP. The abbreviations are the same as those mentioned in Table II.

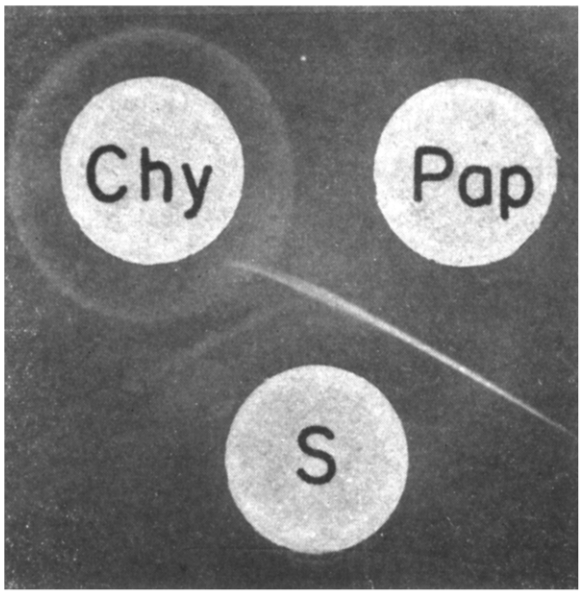

FIGURE 5: Double diffusion in agar plate of antipapain serum (S) with papain (Pap) and chymopapain (Chy).

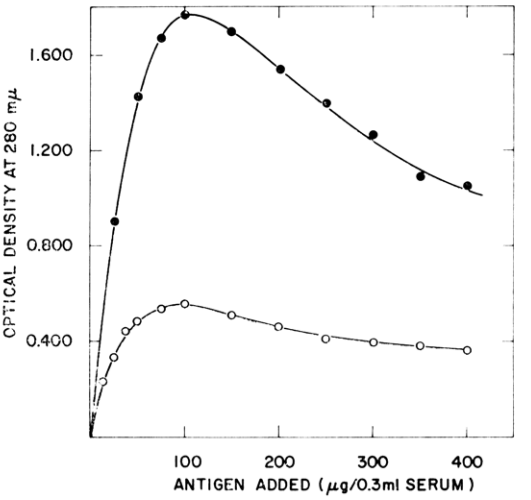

FIGURE 6: Precipitin curve of antipapain serum with papain (●—●) and chymopapain (○—○).

Inactivating Properties of the Various Antibody Preparations. The capacity of the various antibody preparations to inhibit the catalytic activity of papain was measured on substrates of both high molecular weight (casein) and low molecular weight (benzoyl-L-arginine ethyl ester, BAEE). As may be seen in Figure 4, the extent of inhibition for each of these two types of substrate was found to be different. Whereas the proteolysis of casein was inhibited to an extent of up to 85%, the degree of inhibition in the case of BAEE did not exceed 60%. However, all antibody preparations tested possessed identical inhibitory capacity as regards each of the substrates by itself.

Thus, as far as precipitating or inactivating properties of the antibodies are concerned, none of the methods of purification used hitherto provided any means of selective separation.

Antibodies Isolated with Cross-Reacting Immunoadsorbent. Another attempt at the fractionation of antipapain antibodies was made using as immunoadsorbent not an insoluble derivative of papain itself, but of a cross-reacting enzyme, chymopapain. The existence of a cross-reaction between chymopapain and antipapain serum was demonstrated by the gel diffusion technique (Figure 5) as well as by the quantitative precipitin reaction (Figure 6). Using the latter method, it was calculated that chymopapain precipitates 32% of the antipapain antibodies in the serum. The immunodiffusion experiment showed that only part of the antibodies possess specificity toward both enzymes, a strong spur indicating the presence of a considerable portion of antibodies which react only with papain and not with chymopapain. The interaction between chymopapain and antipapain antibodies was further demonstrated by their capacity to inhibit the enzymic activity of chymopapain, as shown in Figure 7.

The method of preparation of the chymopapain immunoadsorbent was identical with that described for the papain immunoadsorbent. A sample of the antipapain IgG fraction was subjected to three consecutive adsorptions with the chymopapain immuno-

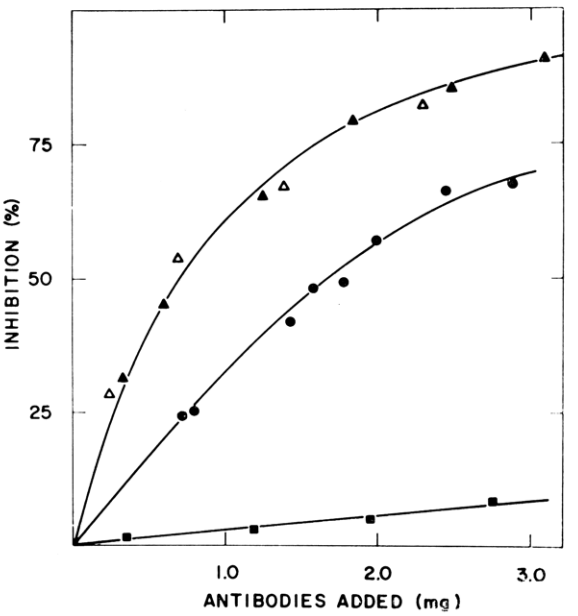

FIGURE 7: Inhibition of enzymatic activity of papain (50 μg) on BAEE by the three species of antibodies. (●——●) Total antibody population (P); (▲——▲) antibodies purified on chymopapain immunoadsorbent (CP); (■——■) antibodies of the PS type (see text for abbreviation; and (△——△) inhibition of chymopapain (50 μg) activity by CP antibodies.

adsorbent. The antibodies eluted after each adsorption step were identical in every respect and were, therefore, pooled. They will be designated in the following as "CP." The antibodies remaining in the supernatant fluid from the adsorption on the insolubilized chymopapain were then adsorbed on papain immunoadsorbent, eluted, and designated "PS." Both the CP and PS antibody preparations were found to be reactive with papain in the precipitin test. The precipitation was carried out with ^{131}I-labeled papain (50 μg) in the region of antibody excess (2 mg). The resultant precipitates were washed and analyzed for their papain content and for their enzymatic activity on BAEE. As shown in Table III, the amount of papain precipitated by the two antibody preparations was essentially the same. In contrast, the enzymic activity of the precipitates from the two preparations was completely dissimilar. Whereas the precipitate obtained with the antibodies isolated with chymopapain (CP) was practically devoid of enzymatic activity, that obtained with the antibodies specific to papain alone (PS) retained almost the full activity of the enzyme, although the amount of antibodies present in the precipitate with the PS antibodies doubles that in the precipitate with the CP antibodies.

TABLE III: Residual Papain Activity in Immune Precipitates of the Two Antibody Species.[a]

Antibody Prepn[b]	Antibody in Ppt (μg)[c]	Papain in Ppt (μg)[d]	Act. in Ppt (μg of papain)[e]	Residual Act. in Ppt (%)
CP	430	29.1	1.5	5.8
PS	920	31.4	29.8	95.0

[a] Each antibody preparation (2 mg) was added to 50 μg of ^{131}I-labeled papain (1160 cpm/μg). [b] CP, antibodies adsorbed with chymopapain immunoadsorbent. PS, antibodies adsorbed with papain immunoadsorbent after exhaustive adsorption with chymopapain immunoadsorbent. [c] Calculated from optical density of the solution of precipitate in 0.1 N NaOH. [d] Calculated from radioactivity of the precipitate. [e] Determined on BAEE.

The extent of inhibition of papain by the two antibody species (CP and PS) was compared quantitatively with that effected by the total antibody population as purified from the IgG fraction directly on papain immunoadsorbent (and designated "P"). The results, shown in Figure 7, demonstrate that CP antibody was a very efficient inhibitor, that PS antibody exhibited hardly any inhibitory capacity, and that the P antibody, which was identical with the preparations described earlier, yielded intermediate inhibition values.

The CP and PS antibody preparations were also tested for their capacity to inhibit the catalytic activity of chymopapain on BAEE. As was to be expected, PS antibody had no inhibitory effect at all on chymopapain, while CP antibody was found to inhibit chymopapain to exactly the same extent as it had inhibited papain (Figure 7).

The interaction in agar gel between the three species of antibody (CP, PS, and P) and both papain and chymopapain is shown in Figure 8. Papain interacts equally well with all three species of antibody, while chymopapain, on the other hand, precipitates with P and CP only and does not interact with PS antibody. The spur at the intersection of the precipitin bands of papain and of chymopapain with the total antibody population (P) is further proof that chymopapain interacts with only a fraction of the antibodies, and that this fraction is identical with the CP species defined above. The CP antibodies gave a band of identity with both enzymes.

Discussion

Enzymes are usually macromolecules of considerable size and complexity, and therefore contain many different antigenic specificity determinants. Hence they are apt to give rise to heterogeneous populations of antibodies with specificities directed toward various groups and arrangements of groups in the enzyme molecule. There is no reason to assume that all or any of these antigenic determinants on the enzyme should include the catalytic site of the molecule. On the other hand, if antibodies should exist whose specificity were directed toward groupings associated with the active center of the enzyme antigen, their reaction with the enzyme would be expected to bring about inhibition of the enzymic activity. Thus the inhibitory capacity of such antibodies could be higher than that of antibodies whose specificity is directed toward other regions of the antigen. The problem then arises whether such antibodies can be selectively separated from the entire antibody population to yield fractions of differing inhibitory capacity.

One approach used in this work for such a selective separation in the papain system was to try to isolate the antibodies specifically directed toward the active site by means of an enzyme derivative in which this site is blocked. Previous work (Arnon, 1965) had indicated that mercuripapain precipitated antipapain very efficiently, but allowed a small fraction of the antibodies, which reacted only with papain, to remain in solution. If the latter antibodies were specific for a determinant including the active sulfhydryl group or adjacent groups, then the antibodies precipitated by mercuripapain should inhibit the catalytic activity of the enzyme to a lesser degree. The inhibitory capacity of these species was nevertheless equal to that of antibodies purified with native papain (Table II, Figure 4).

The use of an immunoadsorbent prepared by reacting papain *via* its sulfhydryl groups with bromoacetylcellulose was equally unsuccessful in selective fractionation of antipapain antibodies (Table II, Figure 4),

even though it could be assumed that such an enzymatically inactive papain derivative would lead to a more efficient adsorption of those antibodies which were directed toward regions of the antigen other than the active site. The different antibody preparations obtained by successive adsorptions and elutions on this immunoadsorbent were identical in precipitating and inhibitory capacity, both with each other and with the heterogeneous total antibody population in the unfractionated serum.

It was concluded that the use of the homologous enzyme for purpose of purification of the antibodies could not lead to selective separation of the different antibody species presumed to be present in the solution. Successful selective isolation of antibodies was achieved by making use of the phenomenon of cross-reaction. A great number of cases have been reported in which enzymes derived from different species or from different organs in the same species could be shown to cross-react with the corresponding antisera (*e.g.*, McGeachin and Reynolds, 1960; Nisselbaum and Bodansky, 1961; Fujio *et al.*, 1962). Cross-reaction has also been demonstrated in the case of penicillinases from two different strains (Pollock, 1956). The extent of precipitation and neutralization by the cross-reacting antibody in these and other examples was lower than that obtained with the homologous antibody. In at least one system, however, antibodies purified with an enzyme preparation from another species have been shown to inhibit the homologous enzyme to a greater extent than the antibodies purified with the homologous enzyme (Marshall and Cohen, 1961). Cross-inhibition by antibodies can take place not only with equifunctional enzymes of different origin, but also with different enzymes having similar catalytic sites. Thus, chymotrypsin is inhibited by antitrypsin antibodies (Arnon and Schechter, 1966). In a similar manner, it was attempted in the present investigation to use chymopapain as a cross-reacting enzyme for antipapain antibodies.

Chymopapain (Jansen and Balls, 1941) is obtained from the papaya latex. Similarly to papain it is a sulfhydryl protease, and although the amino acid sequence around its thiol group is different from that in papain (Tsunoda and Yasunobu, 1966), the two enzymes are quite similar in their substrate specificities (Ebata and Yasunobu, 1962).

As expected, chymopapain was both cross-precipitated and inhibited by antipapain antibodies. It can thus be assumed that the regions in the molecule that these two enzymes have in common include those antigenic determinants, whose interaction with the antibodies is responsible for the decrease in catalytic activity of both enzymes. The gel diffusion experiments (Figure 5) indicated that only a fraction of the antibodies reacted with both enzymes, while the remainder reacted with papain only. Separation of these two types of antibodies was achieved by treatment of the antibody-containing solution with chymopapain immunoadsorbent, followed by binding of the antibodies remaining in the supernatant on papain immunoadsorbent. The first step yielded the antibodies which are common for papain and chymopapain (denoted CP), whereas the second step yielded the strictly papain-specific antibodies (PS).

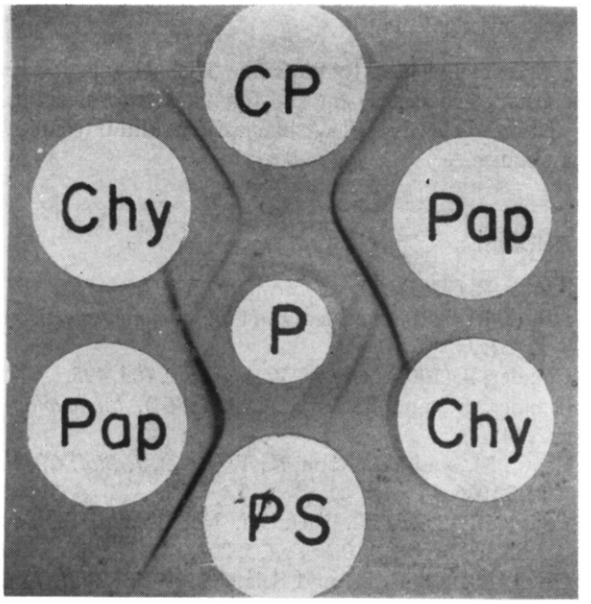

FIGURE 8: Double diffusion in agar gel. P, CP, and PS are the antibody species (for abbreviations see text); Pap, papain; Chy, chymopapain.

The CP antibodies inhibited papain activity on BAEE much more efficiently than the total antibody population, while the PS antibodies, although being more efficient in precipitating with papain had no inhibitory effect at all (the degree of inhibition in antibody excess of different preparations ranged from 1 to 7%, this low value probably being due to contamination with CP antibodies, which had not been completely eliminated by the adsorption with chymopapain). The observed residual activity in the papain–antipapain complexes may, therefore, be regarded as an over-all value expressing the probability of the interaction of papain with the different antibody species which constitute the heterogeneous total antibody population.

The main objective of this study was to determine whether the heterogeneous population of antibodies to an enzyme consists of antibodies which differ in their capacity to neutralize the enzyme, and whether such antibody species can be selectively separated. With this objective achieved, it became possible to study the mechanism of inhibition of both papain and chymopapain, and hence the way is open for the elucidation of the nature of the interaction of these two enzymes with antipapain antibodies (Shapira and Arnon, 1967b). It is hoped that in other related enzymes, such as trypsin and chymotrypsin, where much information is available concerning their primary and secondary structure, a similar study could lead to antigenic mapping of the enzymes.

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