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Immunochemical Studies with Chymotrypsinogen A*

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Summary. Antiserum prepared by immunization of rabbits with chymotrypsinogen A reacted in equal titre with the proenzyme and alpha, beta, delta and gamma chymotrypsin in serological tests. Inactivation of the four enzymes by diisopropylfluorophosphate did not diminish their precipitability. Immunodiffusion tests in gel plates with the proenzyme produced a single precipitin band which merged without spur formation with the bands produced by each of the native and inactivated enzymes. The antiserum partially inhibited activation of the zymogen by trypsin. The proteolytic activity of each of the four chymotrypsins was also inhibited by the antiserum.

INTRODUCTION

Chymotrypsinogen A is a convenient proenzyme for immunochemical investigation because it is available in a highly purified form, gives rise to a series of chymotrypsins upon activation by trypsin, and many of its physical and biochemical qualities have been clearly established.

The first study of chymotrypsinogen A as an antigen was by Ten Broeck (1934), who concluded from anaphylactic experiments in guinea-pigs that this proenzyme was immunologically distinct from chymotrypsin and trypsin despite a number of cross-reactions between the homologous proenzyme–enzyme pair.

As a result of subcutaneous immunization with chymotrypsinogen A in Freund's adjuvant Rickli and Campbell (1963) showed that rabbits produced both antichymotrypsinogen A and antichymotrypsin. The former antibody did not react with alpha chymotrypsin in gel tests, but the latter gave reactions of identity with chymotrypsinogen A and alpha enzyme. It was suggested that proenzyme activation *in vivo* resulted in the formation of the alpha enzyme and thus the antibody.

Investigation of three of the enzymes formed by activation of chymotrypsinogen A (alpha, beta and gamma chymotrypsin) revealed that though they were poor antigens, they were indistinguishable by the precipitin reaction (Northrop, Kunitz and Herriott, 1948). Alpha chymotrypsin also did not sensitize guinea-pigs to systemic anaphylaxis or induce antibody formation in rabbits (Fleming and Riddel, 1961), but the quantity of antigen administered may have been insufficient for the latter purpose.

In this study an antiserum derived from immunization of rabbits with chymotrypsinogen A was tested for it ability to react with several proenzymes and enzymes of the bovine pancreas. The nature of these reactions was examined by the Ouchterlony method. The effect of antiserum on the enzyme activity of alpha, beta, delta and gamma chymotrypsin was examined as well as the ability of the antiserum to retard chymotrypsinogen activation.

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EXPERIMENTAL METHODS

Reagents

Five times recrystallized (5 ×) chromatographically pure chymotrypsinogen A, 3 × alpha chymotrypsin, 2 × beta, gamma chymotrypsin and trypsin, and crystalline delta chymotrypsin and trypsinogen were all products of Worthington Biochemical Corp., Freehold, N.J. Chymotrypsinogen B was a gift of M. Laskowski, Sr., Marquette University. Soybean trypsin inhibitor (5 ×) and Hammersten quality casein were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Diisopropylfluorophosphate was a product of Mann Research Laboratories, New York. Other chemicals were reagent grade.

Immunological Procedures

Chymotrypsinogen A was used as the immunizing antigen for rabbits in an emulsion with Freund's adjuvant administered by foot-pad injection. Subsequent intravenous injections of the proenzyme in saline were given to maintain a high antibody titre. The total amount of proenzyme given was 25–30 mg. per rabbit.

The titre of the antiserum was determined by capillary precipitin tests with an incubation period of 3 hours at room temperature and 18–24 hours at 4° before reading the results. Ouchterlony gel immunodiffusion tests were conducted as previously described for other enzymes (Niece and Barrett, 1963) using undiluted antiserum and antigens at various concentrations.

Biochemical Procedures

Each chymotrypsin was analysed for enzyme activity by a casein digestion procedure (Kunitz, 1947) as modified by Wu and Laskowski (1955). The assay is dependent upon the enzymatic liberation from casein of trichloroacetic acid soluble substances which absorb at 280 mμ as measured in a Beckman DU spectrophotometer.

Alpha, beta, delta and gamma chymotrypsin were treated with diisopropylfluorophosphate (DFP) (Naughton, Sanger, Hartley and Shaw, 1960). Casein hydrolysis assays confirmed the inactivation of the enzymes.

Serological inhibition of the chymotrypsins was examined by adding 6 μg. of the enzyme to be studied to 0·1 ml. of heat-inactivated (56°) antiserum dilution. A 0·1 M borate buffer, pH 8·0, containing 0·01 M calcium chloride was used as the diluent to make a final volume of 1·0 ml. After a 5 minute incubation at 37°, 1·0 ml. of 1 per cent casein was added to initiate the enzyme activity assay. Controls consisted of a replicate of the above in which normal rabbit serum was substituted for immune serum and a chymotrypsin stability control for the enzyme in the absence of serum. Controls on chymotryptic activity of the heated sera and for spontaneous degradation of the substrate were negative.

To determine if the antiserum would retard the tryptic conversion of the proenzyme to delta chymotrypsin (Neurath, Rupley and Dreyer, 1956), 100 μg. of the proenzyme and 0·1 ml. of the heat-inactivated antiserum dilution were adjusted to 0·7 ml. with 0·1 M borate buffer, pH 8·0, and incubated for 5 minutes at 37°. One μg. of trypsin (0·1 ml.) was added and the mixture held in an ice bath for 1 hour. Activation was stopped by the addition of 2 μg. (0·1 ml.) soybean trypsin inhibitor. The tubes were then returned to the 37° water bath, and 0·1 ml. of 0·1 M $CaCl_2$ and 1·0 ml. of casein solution were added. This mixture was then analysed for casein digestion as before. Controls again included replacement of immune serum by normal serum, a proenzyme activation control containing only proenzyme, trypsin and trypsin inhibitor and a trypsin inhibitor control in which

proenzyme was omitted to ensure that any recovered enzyme activity was not due to excess trypsin or insufficient trypsin inhibitor. To confirm that an excess of antibody did not quench the activity of any enzyme as it was formed an additional control containing 6 μg. of delta chymotrypsin added to the proenzyme–antibody mixture was prepared. Recovery of enzyme activity in this control verified that enzyme formed could in fact be detected. The usual casein, serum and proenzyme blanks were also employed and were negative.

RESULTS

Precipitin tests in fluid medium with the immune serum yielded the same titre (within a two-fold dilution) with the proenzyme as with the specific chymotrypsins derived from it (Table 1). No differences were detected between the native and DFP-inactivated enzymes. Two other proenzymes, chymotrypsinogen B and trypsinogen, were not precipitable nor was trypsin. Tests with trypsin were considered important in relation to the activation studies.

TABLE 1

SPECIFICITY OF THE IMMUNE SERUM AS DETECTED BY PRECIPITIN TESTS

	Antigen concentration (μg./*ml.*)				
	20	10	5	2·5	1·25
Chymotrypsinogen A	+	+	+	+	−
Alpha chymotrypsin	+	+	+	+	−
Alpha chymotrypsin–DFP*	+	+	+	+	−
Beta chymotrypsin	+	+	+	+	−
Beta chymotrypsin–DFP*	+	+	+	+	−
Delta chymotrypsin	+	+	+	+	−
Delta chymotrypsin–DFP*	+	+	+	+	−
Gamma chymotrypsin	+	+	+	−	−
Gamma chymotrypsin–DFP*	+	+	+	−	−
Chymotrypsinogen B	−	−	−	−	−
Trypsin	−	−	−	−	−
Trypsinogen	−	−	−	−	−

* Refers to diisopropylfluorophosphate treated enzyme. Controls with normal rabbit serum were negative.

\+ = precipitation, − = no precipitation.

To determine if multiple antigen–antibody systems were responsible for the extensive cross-reaction seen in the precipitin tests, double gel diffusion analyses according to Ouchterlony were performed. Artifactual bands known to be produced by alpha chymotrypsin (Niece and Barrett, 1963) were avoided by the use of low concentrations of the enzyme. In each instance the single band produced by the antibody and its homologous antigen merged (reaction of identity) with that of the enzyme tested (Fig. 1) regardless of whether the enzyme was active or not. Chymotrypsinogen B, trypsinogen and trypsin failed to react in the gel tests.

In the first experiments designed to ascertain whether the antiserum inhibited the activity of the chymotrypsins untreated sera were used. These experiments were unsuccessful apparently because of a slightly heat-labile chymotrypsin–trypsin inhibitor present in the sera (McCann and Laskowski, 1953; Peanasky and Laskowski, 1953). Use of heat-inactivated sera avoided this problem and the results of such experiments are presented in Table 2. The results were similar regardless of the enzyme considered; a 1:50 dilution

of the antiserum abolished all enzyme activity and a 1:250 dilution produced inhibitory activity ranging from 25 to 46 per cent. Heat-inactivated normal serum diluted 1:250 had little effect on the chymotrypsins but a 1:50 dilution diminished activity about 50 per cent.

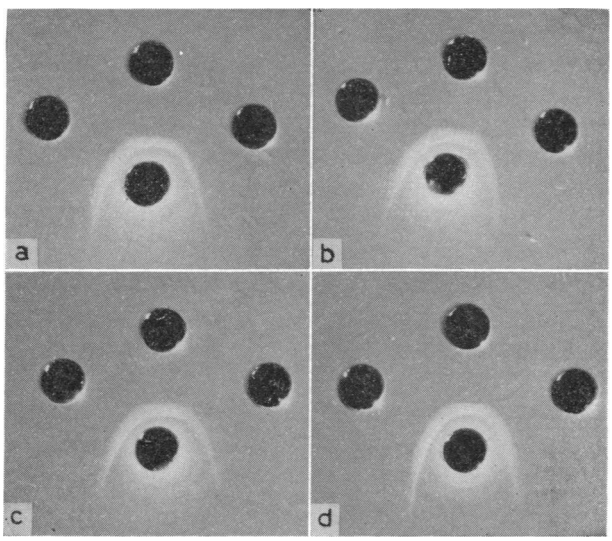

FIG. 1. Immunodiffusion tests with the proenzyme, native and DFP-treated chymotrypsins. Magnification *c.* $\times 1\frac{1}{2}$. Reagents: Lower centre well, undiluted antiserum, all slides; upper centre well, chymotrypsinogen A, 500 µg./ml. all slides; left well, DFP-treated chymotrypsins, a = alpha, b = beta, c = delta, d = gamma, 625 µg./ml.; right well, native chymotrypsins, a = alpha, b = beta, c = delta, d = gamma, 625 µg./ml.

TABLE 2

ANTIBODY INHIBITION OF CHYMOTRYPSINS

	Serum dilution	*Optical density of enzyme assay**		
		Normal serum	*Immune serum*	*Enzyme only*
Alpha chymotrypsin	1 : 50	0·162	−0·003	0·325
	1 : 250	0·278	0·243	
Beta chymotrypsin	1 : 50	0·164	−0·011	0·324
	1 : 250	0·271	0·175	
Delta chymotrypsin	1 : 50	0·112	0·007	0·228
	1 : 250	0·196	0·148	
Gamma chymotrypsin	1 : 50	0·159	−0·011	0·327
	1 : 250	0·298	0·207	

* Average of triplicate determinations of enzyme activity upon the casein substrates as described in the text.

Activation of chymotrypsinogen by trypsin to yield delta chymotrypsin was conducted in the presence of normal and immune serum. Both sera were active inhibitors of this transformation when diluted 1:7 (Table 3), again apparently because of the chymotrypsin–trypsin inhibitor in the sera which effectively neutralized the added trypsin. A 1:35

dilution of the sera overcame this effect allowing the specific, almost total inhibition by the antiserum to be demonstrated. The small amount of activity recovered may have been due to trypsin, since the trypsin inhibitor was but 99 per cent effective. The proenzyme activation control in the absence of serum indicated formation of 10 μg. of delta chymotrypsin. This figure was determined from the activity of the mixture compared to standard

TABLE 3

SEROLOGICAL INHIBITION OF THE ACTIVATION OF CHYMOTRYPSINOGEN A

Serum dilution	*Optical density of enzyme assay**	
	Normal serum	*Immune serum*
1 : 7	0·044	0·052
1 : 35	0·827	0·053

* Average of triplicate determinations of enzyme activity in a mixture containing 100 μg. chymotrypsinogen A, 0·1 ml. of serum dilution and 1·0 μg. trypsin. After incubation for 1 hour at 0° 2 μg. soybean trypsin inhibitor were added and the mixture analysed for casein digesting ability. Controls: 1. Activation of 10 μg. chymotrypsinogen A in the absence of serum. 2. Inhibition of trypsin by soybean trypsin inhibitor, 99 per cent. 3. Inhibition of added delta chymotrypsin, 60 per cent. Correction applied.

curves of delta chymotrypsin enzyme activity on the casein substrate. The control for excess antibody resulted in a 60 per cent inhibition of the added 6 μg. of enzyme by a 1:35 dilution of antibody; consequently, the enzyme activity in the presence of this amount of antiserum has been corrected.

DISCUSSION

The participation of the four chymotrypsins in serological precipitation reactions is evidence of multiple antigenic determinant sites on these molecules since monovalent antigens do not react with antibody to produce precipitates. The chymotrypsins apparently have only one enzymatically active site involving a single serine residue (Balls and Jansen, 1952; Schaffer, May and Summerson, 1953), a single histidine residue (Schoellmann and Shaw, 1963) and a methionine residue (Koshland, 1963); it may therefore be concluded that one or more antigenic sites are distinct from the enzymic centre. Since the diisopropylfluorophosphate treated enzymes were as reactive with antibody as were the native enzymes it is suggested that the serine of the enzymic centre is not a part of an antigenic site.

Chymotrypsinogen was immunologically equivalent to alpha, beta, delta and gamma chymotrypsin on the basis of capillary precipitin tests. To examine this more critically, the proenzyme was compared by Ouchterlony tests with each of the chymotrypsins. In every instance the precipitin band produced by the proenzyme merged in a reaction of identity with the precipitin bands of the several enzymes. This is different from the results of the pepsinogen–pepsin (Schlamowitz, Varandani and Wissler, 1963) and procarboxypeptidase A–carboxypeptidase A (Barrett, 1965) system. For both of these proenzyme–enzyme pairs the antiproenzyme reacted in partial identity with the enzyme. With the

former systems these results are not unexpected since the activation of pepsinogen to pepsin and procarboxypeptidase A to carboxypeptidase A represent a change in molecular weight from 42,000 to 36,400 (Bovey and Yanari, 1961) and 96,000 to 34,000 (Neurath, 1961) respectively. Such gross changes in molecular weight were detectable by the anti-proenzyme. However, the activation of chymotrypsinogen A through the chymotrypsin series (pi, delta, alpha, beta and gamma in order of formation) occurs with little alteration in molecular weight as presented in the following scheme (Desnuelle, 1961; Laskowski, 1961):

1. chymotrypsinogen A	trypsin ⟶	pi chymotrypsin
2. pi chymotrypsin	trypsin ⟶	delta chymotrypsin plus serylarginine
3. delta chymotrypsin	trypsin ⟶	alpha chymotrypsin plus threoninyl-asparagine
4. alpha chymotrypsin	trypsin ⟶	beta and gamma chymotrypsin plus unknown products

These minor changes in molecular weight may represent such minor changes in molecular and antigenic structure, at least to the alpha enzyme level, that they could easily escape detection by antibody and be the reason for the reactions of identity observed here.

Enzyme inhibition studies with antibody do not always determine whether the antigen sites are identical with the enzymatic groupings since the antibody–enzyme interaction can prevent substrate attachment through steric hindrance or by alteration of the polar characteristics of the enzyme (Cinader, 1957). Consequently our primary interest in the inhibition studies is summarized in the findings that all four enzymes examined were inhibited by the antibody and to the same extent. A 5 minute preincubation of a 1:50 dilution of antibody abolished all enzyme activity and a 1:250 dilution still produced some specific inhibition beyond that of normal serum.

Comparison of the delta chymotrypsin formed in the activation study with standard delta enzyme assays indicated that approximately 2 μg. of enzyme were produced from 100 μg. of proenzyme in the presence of antiserum (1:35). That this inhibition of the tryptic conversion of chymotrypsinogen to delta chymotrypsin was not a result of a trypsin–antiserum interaction was confirmed by the failure of trypsin to react with the antibody in precipitin tests although antigen–antibody combination may have occurred in the absence of precipitation. Non-conversion by trypsin was not due to excess antibody since the figures reported are corrected for this effect.

Throughout this report care has been taken to avoid the term antichymotrypsinogen. As Rickli and Campbell (1963) have suggested, activation of the proenzyme to an enzyme could have occurred during the immunization process resulting in the formation of an antichymotrypsin. But one is then confronted with the dilemma of having to determine which antienzyme(s) was produced—anti-pi, anti-delta, anti-alpha, etc. The antiserum described here reacted identically in gel tests with all four of the enzymes tested as well as the proenzyme and there was no possibility of distinguishing between the enzymes by this method. In fact, as already suggested, the close structural relationship of these antigens may place them beyond the limit of recognition by a single antiserum. Further support for this suggestion is derived from the comparison of the native and DFP-treated

enzymes, which reacted identically with the antiserum. Our antiserum could not distinguish the differences in structure associated with the addition of diisopropyl moieties to serine residues in the enzymes. Since the major difference between the native chymotrypsins is the removal of *terminal* dipeptides, which do not extensively alter the structure of the molecules and reduce their molecular weight by only 200, the recognition of one chymotrypsin and not another would require a very specific antibody. Rickli and Campbell have labelled one antibody which was produced by immunization with chymotrypsinogen A, as an anti-alphachymotrypsin without evidence concerning any reactions of the antiserum with other chymotrypsins. Since alpha chymotrypsin, like chymotrypsinogen A, is susceptible to further modification to the beta and gamma enzymes *in vivo*, perhaps immunization with the latter is the best approach to a solution of this problem.

The failure of the antichymotrypsinogen antibodies described by Rickli and Campbell to react with the alpha enzyme is the first description of an antiproenzyme which failed to react with the corresponding enzymes (Arnon and Perlmann, 1963; Schlamowitz *et al.*, 1963; Van Vunakis, Lehrer, Allison and Levine, 1963; Barrett, 1965). An immediate explanation for this is not apparent.

Chymotrypsinogen A is the third proenzyme to be compared immunologically with its enzyme counterparts. As with pepsinogen (Arnon and Perlmann, 1963; Schlamowitz *et al.*, 1963; Van Vunakis *et al.*, 1963) and procarboxypeptidase A (Barrett, 1965) an antibody can be produced which inhibits activation of the proenzyme to an enzymically active form. Inhibition of the catalytic functions of the enzyme is also an effect of the antibody. Unlike the latter systems identification of the antibody responsible for these effects is made difficult by the close structural relationship of the antigens involved and their potential alterations *in vivo*.

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