AN IMMUNOLOGICAL APPROACH TO THE STUDY OF EVOLUTION OF TRYPSINS*

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Abstract.—Antibodies were prepared in rabbits against trypsins obtained from four different species—namely bovine, porcine, spiny Pacific dogfish, and starfish (Evasterias trochelii). Each of the antisera, or the immunoglobulin G fraction thereof, was tested for its capacity to react with each of the four enzymes. The immunological reaction was assessed by three different techniques—precipitin reaction, antigen binding capacity, and inhibitory effect of the antibodies on the proteolytic activity of the enzymes. In each case, the homologous enzyme gave the strongest reaction with its antiserum but the heterologous enzymes were also capable of reacting to an appreciable extent. The cross-precipitation was the least sensitive method, and gave relatively low values, whereas cross-inhibition and cross-binding of radioactively labeled antigens indicated high extent of cross-reaction between the various trypsins. The relative capacity of interaction of the four enzymes with the four antibodies could be related to the order in which these enzymes developed during evolution. Thus the "order" of similarity was bovine > porcine > dogfish > starfish.

The immunological approach has been extensively applied in recent years to the study of enzymes. One of the most fascinating problems that can be approached by immunological methods relates to biochemical evolution.1 Antibodies to specific enzymes can be useful to the search for enzymes of biochemical pathways which disappeared in the course of evolution, or to detect similarities among enzymes that have persisted through the ages. In many cases the evolutionary changes that occur in the molecular composition and structure may be very extensive although the catalytic activity and specificity are barely affected. By studying the immunological interactions of analogous enzymes isolated from different species one may expect, therefore, cross inhibition by the antibodies concomitant with the immunological cross-reaction. And, just as immunological cross-reaction indicates similarity between antigenic determinants, the cross-inhibition may be taken to imply similarity in the structure of the catalytic center or adjacent sites on the enzymes.

Studies reported in the literature have for the most part been concerned with two types of relationships. On the one hand, enzymes that originate from different organs of the same species were compared for their capacity to react with the corresponding heterologous antisera. McGeachin and Reynolds,2 for example, have studied amylases from hog pancreas, saliva, and liver, and have concluded that the liver amylase must be distinctly different from the other amylases. Similar studies have been performed with human lactic dehydrogenases from liver and heart.3-4

On the other hand, studies have been conducted to compare isofunctional
enzymes from different species: Penicillinases from different strains of *Bacillus cereus* were compared according to their relative inactivation by the corresponding antisera, carbamyl phosphate synthetase from frog and rat were compared by measuring the extent of their inhibition by the anti-frog enzyme, and hen and duck egg white lysozyme were found to show appreciable extent of similarity as manifested by both cross-precipitation and cross-inhibition tests.

The availability of trypsins from various sources including those of two higher mammals, the bovine and the porcine, and two lower vertebrates, the spiny Pacific dogfish and starfish (*Evasterias trocheli*), combined with the fact that the amino acid sequence of some of them is wholly or partially known, prompted us to compare their mutual immunological reactivity.

**Materials and Methods.**—Bovine trypsin (crystallized, lot 44-14) was obtained from Worthington; porcine trypsin (dialyzed and lyophilized batch no. 640426-2) was purchased from Novo Industries A/S; dogfish trypsinogen A was a gift from Dr. Royce Haynes; and starfish trypsin B was a gift from Dr. William P. Winter. Partially purified human trypsin was a gift from Dr. Patricia J. Keller.

Labeling of the enzymes was carried out with *I* (New England Nuclear Corp.). Enzymatic activity was assayed with casein as substrate.

**Immunological procedures:** Immunization of rabbits with each of the four enzymes mentioned above was performed by injecting 5–10 mg protein in complete Freund’s adjuvant (Difco) into multiple intradermal sites. The sera, collected from the marginal ear vein were used for precipitin tests, and whenever the antibody titer decreased an intramuscular booster injection was administered.

Immunoglobulin G (IgG) fraction was isolated from the antisera by precipitation with 40 per cent saturated ammonium sulfate, followed by 3–4 successive precipitations with 33 per cent saturated ammonium sulfate. Antigen-binding capacity experiments were performed with *I* labeled enzymes. The antibodies were also tested for their capacity to inhibit the catalytic activity of the various trypsins toward casein.

**Results.**—Trypsins obtained from three different sources—namely bovine, porcine, and starfish, and trypsinogen A obtained from the spiny Pacific dogfish were each used for the immunization of 2 or 3 rabbits. Preimmune sera of all the rabbits did not precipitate with any of the four materials investigated, whereas appreciable precipitin titers were observed in all immune sera.

The immunological interrelationships between the trypsins of the four species were studied by means of three different approaches. Firstly, the homologous precipitin reactions and the cross precipitations of the four enzymes were performed with each of the four antisera; secondly, the capacity of the various antibodies to inhibit the catalytic activity of the four enzymes was compared; and thirdly, the relative capacity of each of the four antibodies to bind the four radioactively labeled enzymes was measured. In the following, results are presented for each antibody separately. The precipitin reactions are plotted as amounts of precipitate obtained from a given volume of the fractionated IgG solution; the results of the antigen-binding and the inhibitory capacity experiments are plotted versus the calculated amounts of precipitating antibodies in the IgG solution used; this measure was taken in order to compensate for differences in antibody titers of the different antisera.

Figure 1 depicts the interaction of anti-bovine trypsin with the trypsins of the four species. In the precipitin reaction (Fig. 1A) the porcine enzyme could pre-
Precipitate almost 30 per cent of the antibodies, whereas the dogfish enzyme precipitated only about 15 per cent and the starfish enzyme did not precipitate at all. On the other hand, the relative capacity of these antibodies to bind the heterologous enzymes (Fig. 1B) was very significant. Although the homologous antigen was bound more efficiently than the other enzymes, the differences were relatively small. Similarly, the capacity of the antibodies to inhibit the proteolytic activity of the heterologous enzymes (Fig. 1C) was very high. Porcine trypsin was inhibited essentially to the same extent as the homologous antigen, and the inhibitory effect of the antibody, even on dogfish and starfish trypsins, was more than 50 per cent of its effect on the bovine enzyme.

Figure 2 demonstrates the interactions with anti-porcine trypsin. Again, in all three techniques used for evaluation of the immunological reaction, the homologous porcine trypsin showed the highest capacity to react with the antibodies. The extent of cross-precipitation of the antibodies with the trypsins from other sources was relatively low, bovine trypsin being the best cross-reactant (Fig. 2A). On the other hand, the extent to which these antibodies bound the radioactively labeled trypsins (Fig. 2B), and the relative inhibitory capacity of the proteolytic activity of the heterologous enzymes (Fig. 2C) was remarkably high. It is noteworthy that in the antigen-binding experiment the starfish trypsin appears to be more tightly bound than the dogfish trypsinogen, although in the two other techniques the dogfish enzyme seems to react with the antibodies more efficiently.

A similar set of phenomena was observed with the dogfish trypsinogen and the starfish trypsin systems, as demonstrated in Figures 3 and 4. The interactions with the anti-dogfish trypsinogen (Fig. 3A, B, and C) clearly indicate that the starfish enzyme is the best cross-reactant among those studied, in all three techniques used, and that the bovine enzyme is the least reactive. With the anti-starfish trypsin, on the other hand (Fig. 4A, B, and C), the bovine and the porcine enzymes are shown to be approximately equally weak cross-reactants and the dogfish enzyme is the only enzyme which gives an appreciable extent of interaction.
Human trypsin, in a partially purified state, was available in a small quantity and was tested for its capacity to cross precipitate with the four antisera investigated. The only serum which showed cross-reaction was the anti-bovine trypsin serum, whereas the other three antisera were completely unreactive. The extent of cross-reaction with the anti-bovine trypsin was approximately 30 per cent of the homologous reaction and is shown in Figure 1A, in comparison to the reaction of these antibodies with the other trypsins.

Discussion.—Among the four protein antigens used in this study three were trypsins from various sources, whereas the fourth one was a zymogen, trypsinogen. Since, in a previous study, it was shown that in the case of bovine trypsin the enzyme and the zymogen are immunologically indistinguishable, the comparison of the dogfish trypsinogen and its specific antiserum with the trypsins of other species and their corresponding antisera appeared justifiable.

In the reactions of all the antisera tested, the extent of cross-precipitation at
FIG. 4—(A) Precipitin curves of bovine trypsin (●), porcine trypsin (▲), dogfish trypsinogen A (■), and starfish trypsin (○) with the immunoglobulin G fraction (A280 = 22) of anti-starfish trypsin. (B) Binding of 125I-labeled bovine trypsin (●), porcine trypsin (▲), dogfish trypsinogen A (■), and starfish trypsin (○) to anti-starfish trypsin. The amounts of antibodies are based on the precipitin test. (C) Inhibition of proteolytic activity of bovine trypsin (●), porcine trypsin (■), dogfish trypsin (▲), and starfish trypsin (○) by anti-starfish trypsin. The amounts of antibodies are based on the precipitin test.

best reached 30 per cent of the homologous precipitin reaction, and in most cases it was 15 per cent or even lower. These results are contrasted by the high extent of cross-inhibition by the antibodies and their high capacity of binding of the heterologous trypsins, which are clear indications for immunological interactions. The basic difference between the precipitin reaction and the antigen-binding is that only one binding site is required for the latter, whereas the precipitation requires at least two (and possibly more) antigenic determinants that can react simultaneously with the antibodies. It must be concluded, therefore, that in those cases where low values of cross-precipitation were observed, the number of sites on the enzymes which are involved in the cross-reaction is small.

Cross-inhibition by antibodies may be the result of a direct interaction with the active site of the heterologous enzyme and as such, reflects the similarity between the active sites of the two enzymes being compared. The higher the extent of inhibition, the closer the resemblance. However, since large protein molecules were used as substrate for the assay of catalytic activity, this conclusion does not necessarily follow. As has been shown with the papain-antipapain system, inhibition by the antibody of the enzymatic activity toward high molecular weight substrate was due chiefly to steric hindrance caused by interactions at regions other than the active site. Hence, cross-inhibition is a general indica-

Table 1. Inhibitory capacity of proteolytic activity by the antibodies to trypsin of different species.*

<table>
<thead>
<tr>
<th>Antibody to</th>
<th>Bovine trypsin</th>
<th>Porcine trypsin</th>
<th>Dogfish trypsin</th>
<th>Starfish trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine trypsin</td>
<td>1.0</td>
<td>1.2</td>
<td>2.86</td>
<td>1.85</td>
</tr>
<tr>
<td>Porcine trypsin</td>
<td>1.75</td>
<td>1.0</td>
<td>2.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Dogfish trypsin</td>
<td>3.28</td>
<td>2.6</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Starfish trypsin</td>
<td>N.D.†</td>
<td>7.4</td>
<td>3.4</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*The numbers represent the relative concentration of antibodies that bring about 50% inactivation of 10 μg enzyme. In each case, the concentration of the homologous antibodies is taken as 1.0. Increase in the value reflects, therefore, a decrease in inhibitory efficiency.
†The inhibition did not reach 50% in the antibody concentration range that was used.
tion of similarity and not necessarily an exclusive measure of similarity in functional regions alone.

In all the immunological systems studied here, the homologous enzyme always gave the strongest interaction with its antiserum by all criteria used. If the relative activity of each antiserum with each of the trypsin antigens is compared (Table 1), conclusions concerning the order of relatedness of the four trypsins can be drawn. For example, bovine trypsin appears to be much closer to porcine trypsin than to the other two, whereas porcine trypsin seems to be close both to bovine and to porcine trypsin, but quite remote from the starfish enzyme. The starfish trypsin appears to bear high relationship to the dogfish enzyme and small resemblance, if any, to both mammalian trypsins. By the same token, the dogfish enzyme, although closest in its reactions to the starfish enzyme, is closer to the porcine trypsin than to the bovine species.

From this accumulated evidence it may be concluded that the "order" of similarity in which these enzymes can be arranged is bovine > porcine > dogfish > starfish. Human trypsin, available in a partially purified state, cross-reacted only with anti-bovine trypsin (Fig. 1A), and shewed no reaction with any of the other antisera, an observation which would place the human before the bovine in the above-mentioned sequence. In each case the high degree of cross-reactivity indicates that many common features, present on the surface of the enzyme, are found in all four proteins. Although these conclusions still await corroboration by more direct approaches, particularly proof of homology of primary structure, they are suggestive of a high degree of similarity in three-dimensional structure possessed by these enzymes, thus establishing the development of trypsins many millions of years before the appearance of men, and its evolution through hundreds of species.

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‡ Abbreviation used: IgG-Immunoglobulin G.

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