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Detection of Distant Antigenic Relationships between Insect and Bird Lysozymes by ELISA

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Summary. We used an indirect enzyme-linked immunosorbent assay (ELISA) for measuring the immunological cross-reactivities between bird lysozymes and a lysozyme isolated from the blood of the insect *Locusta migratoria*. The degrees of cross-reactivity among five avian lysozymes measured by ELISA agreed approximately with those observed in earlier work using microcomplement fixation tests. This latter technique is not suitable for detecting immunological cross-reactivity between proteins that differ in sequence by more than 30%–40%. In contrast, ELISA is able to detect distant relationships between antigens such as lysozymes that differ in sequence by as much as 60%. It seems likely that the use of ELISA procedures will extend the range of homologous proteins that can be compared by immunochemical means.

Key words: Lysozyme — Enzyme immunoassay — Antigenic cross-reactivity — ELISA —Locust

Introduction

Studies with several protein families have shown that the extent of immunochemical cross-reactivity between individual proteins can be used to measure the degree of sequence similarity between them (Prager and Wilson 1971a; Champion et al. 1974, 1975). The microcomplement fixation technique is used most often for these comparisons, and because this technique is very sensitive to amino acid substitutions in the antigen, it is usually not possible to compare proteins that differ in amino acid sequence by more than 30%. However, in many families of homologous proteins, individual members may differ in sequence by a much larger percentage, and in such a case other immunochemical techniques are needed to assess the degree of serological cross-reactivity between them. In the case of the tobamoviruses, for instance, precipitin tests were used to determine the serological relationships, although the coat proteins of the individual members of the group differ in sequence by as much as 60% (Van Regenmortel 1975; 1982; Meshi et al. 1983). Recently, it was found that very weak antigenic cross-reactivity between tobamoviruses could be detected also by an indirect enzyme-linked immunosorbent assay (ELISA) procedure (Van Regenmortel and Burckard 1980), a technique that is much more economical in its use of the different reagents than are precipitin tests. This ELISA procedure allowed cross-reactions to be observed not only using whole virions but also when viral protein subunits of 175 kilodaltons were compared. The ability of ELISA to detect such weak cross-reactivity prompted us to examine whether the same technique would be able to detect a serological relationship between an insect lysozyme and bird lysozymes c.

Insects have been shown to possess in their hemolymph an inducible enzyme that is responsible for humoral immunity and resembles in many properties the lysozymes found in birds (Powning and Davidson 1973, 1976). Amino acid analysis of lysozymes from three species of Lepidoptera showed considerable similarities between the compositions of the insect lysozymes and that of hen lysozyme (Jollès et al. 1979). Partial sequence analysis over the N-terminal 33–38 amino acid residues showed that the insect lysozymes share about 35%–40% identical residues with chicken and human lyso-

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zymes (Jollès et al. 1979; Hultmark et al. 1980). If, by extrapolation to the entire molecule, one assumes that insect and bird lysozymes differ in their sequences by about 60%, it is clear that the microcomplement fixation technique would not be expected to reveal any antigenic cross-reactivity between them. Furthermore, Croizier and Croizier (1978), using antisera prepared against two insect lysozymes, were unable by immunodiffusion and Laurell electrophoresis to detect any cross-reaction between hen egg white and insect lysozymes.

The present report concerns the antigenic properties of a lysozyme isolated from the blood of *Locusta migratoria* (Zachary and Hoffman 1984). This substance of 16.5 kilodaltons can be considered a true lysozyme according to the criteria of Jollès (1969). The existence of an antigenic relationship between this lysozyme and the lysozymes c of different bird species was tested by indirect ELISA. The results show that ELISA is able to detect antigenic relationships between the different lysozymes, and that this technique may extend the range of homologous proteins that can be compared by immunochemical means.

Materials and Methods

Egg Whites and Lysozymes c. Lyophilized egg whites from Japanese quail, turkey, and two forms of duck (A and B) were kindly provided by Dr. Ellen Prager, Berkeley, California, and correspond to the materials used by Prager and Wilson (1971a). Purified chicken lysozyme was commercially available (Merck, Darmstadt. FRG); some experiments were done with lyophilized egg white from freshly laid hen eggs. An aliquot of each egg white was resuspended in distilled water and centrifuged for 30 min at 25,000 rpm. The lysozyme content of the egg whites was estimated as described by Hultmark et al. (1980) by comparison of their lytic activity against a suspension of *Micrococcus luteus* with that of purified chicken lysozyme as a reference.

Insect lysozyme was purified from normal adult locust (*Locusta migratoria*, Orthoptera) serum after coagulation. A good degree of purity was obtained by submitting the acidified heated serum to cation exchange chromatography. The purified material revealed a single stained band in acidic as well as in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Zachary and Hoffmann 1984).

Antisera. Lyophilized rabbit antisera against purified lysozymes of chicken, turkey, Japanese quail, duck A, and duck B were a gift from Dr. Ellen Prager. The sera were from rabbits 417, 427, 429, 435, and 439 respectively (Prager and Wilson 1971a) and were reconstituted in distilled water and centrifuged before use. The bleedings used were the 4th (for antisera to duck lysozymes), 8th (for antisera to turkey and quail lysozymes), and 12th (for antiserum to chicken lysozyme).

Antibodies to locust lysozyme were obtained by immunizing rabbits (at 3- to 4-week intervals) by means of a series of eight intramuscular injections of 250 μg purified lysozyme dissolved in 0.5 ml 0.9% NaCl and 0.5 ml incomplete Freund's adjuvant. The rabbits were bled at monthly intervals. The antiserum used in our experiments was obtained 2 weeks after the last injection.

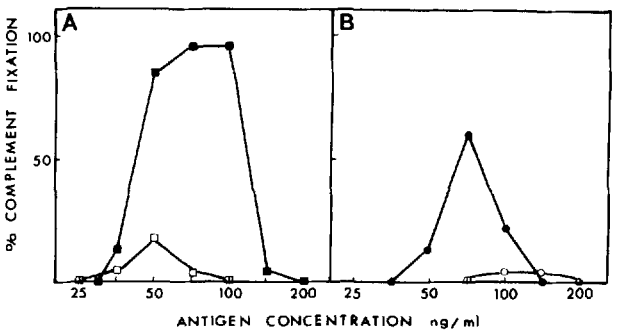

Fig. 1A,B. Microcomplement fixation curves: reactivity of chicken lysozyme antiserum diluted 1/2500 (■ and ●) or 1/5000 (□ and ○) **A** with purified chicken lysozyme and **B** with turkey lysozyme

Complement Fixation Studies. For these studies, one aliquot of each serum was heated at 56°C for 30 min. The experiments were performed according to the method described by Champion et al. (1974) and Van Regenmortel (1982). We omitted ovalbumin in the buffers, because even highly purified ovalbumin always contains traces of lysozyme that would have interfered with the tests.

Indirect ELISA Tests. These tests were performed as described by Van Regenmortel and Burckard (1980), with slight modifications. Disposable polystyrene microtiter plates (Dynatech M 129 B) were used. The wells were coated by incubation for 3 h at 37°C with 250 μl diluted lysozyme in 0.05 M sodium carbonate, pH 9.6, in a concentration range between 0 and 1600 ng/ml. After rinsing three times with PBST (phosphate buffered saline, pH 7.4, containing 0.05% Tween 20), the wells were saturated with 300 μl 1% bovine serum albumin in PBST overnight at 4°C. After a new rinsing, the antibodies, diluted in PBST, were added to the wells and incubated for 2 h at 37°C; the wells were then rinsed as before. Next, 250 μl of goat anti-rabbit globulins conjugated with alkaline phosphatase in PBST were added and incubated for 2–4 h at 37°C. After a last rinsing with PBST, 250 μl of the substrate, p-nitrophenyl phosphate, at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8, were added for incubation times ranging between 45 min and 2 h at 37°C. The absorbance was measured at 405 nm.

Results

In control experiments, the reactivities of several bird lysozyme antisera with respect to homologous and heterologous antigens were measured by the microcomplement fixation method. Results of one such test performed with chicken lysozyme antiserum are presented in Fig. 1. At 1/2500 dilution of antiserum, the chicken lysozyme gave 95% complement fixation, whereas the heterologous turkey antigen produced only 60% fixation. In several such experiments, we were able to reproduce the data on cross-reactivity between the different bird lysozymes described by Prager and Wilson (1971a). No complement fixation was observed when the insect lysozyme was tested against antisera to the bird lysozymes.

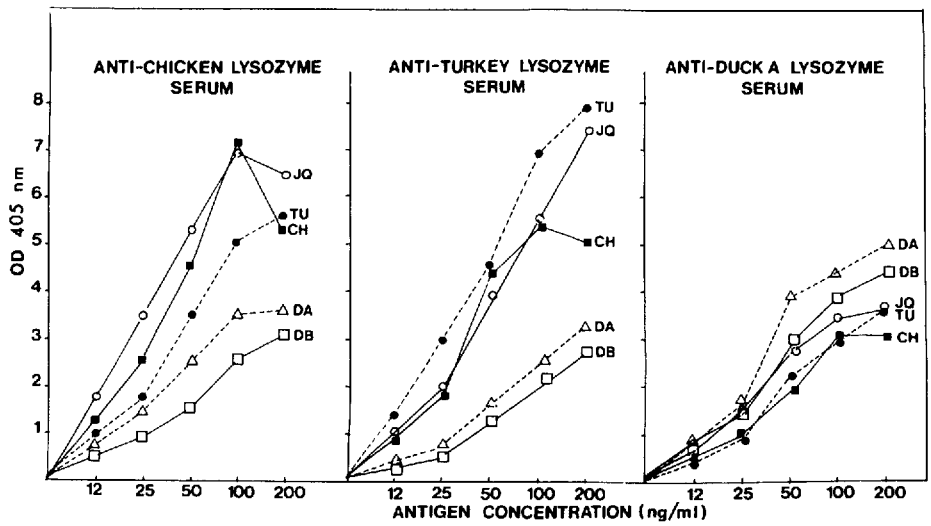

Fig. 2. Indirect ELISA with three bird lysozyme antisera. Coating was done with lysozymes from chicken (CH), turkey (TU), Japanese quail (JQ), duck A (DA), and duck B (DB). The antisera were diluted 1/5000, goat anti-rabbit conjugate was at 1/2000, and the substrate hydrolysis was performed for 45 min at 37°C

Table 1. Comparisons of bird lysozymes by ELISA and microcomplement fixation tests

Lysozyme antiserum source	Lysozyme antigen source	Immunological distance units by complement fixation[a]	Percentage cross-reactivity by ELISA[b]	Number of sequence differences[a]
Chicken	Quail	15	97	6
Chicken	Turkey	15	69	7
Chicken	Duck A	54	49	21
Turkey	Chicken	21	76	7
Turkey	Quail	29	79	10
Turkey	Duck A	60	37	21
Duck A	Quail	116	~79	24
Duck A	Turkey	78	~66	21
Duck A	Chicken	80	~70	21

[a] Data from Prager and Wilson (1971a) and Kondo et al. (1982)
[b] Calculated from Fig. 2 at an antigen concentration of 100 ng/ml, using the optical density obtained with the homologous antigen as 100%. The correlation coefficient relating percentage cross-reactivity to immunological distance for all nine pairs is −0.24 (slope, −0.12) while for the first six pairs it is −0.89 (slope, −0.97)

The suitability of the indirect ELISA procedure for measuring the cross-reactivities between bird lysozymes was then determined. Some of the results obtained with antisera to chicken, turkey, and duck-A lysozymes are presented in Fig. 2. These experiments were performed with egg whites and the concentration of lysozyme in each assay could thus be estimated only roughly. The results obtained with the three antisera diluted 1/5000 show that the degrees of cross-reactivity among the five lysozymes measured by ELISA are roughly proportional to the immunological distances determined previously by microcomplement fixation (Table 1).

We then examined if the ELISA technique would succeed in demonstrating the presence of cross-reactivity between the bird lysozymes and the locust lysozyme. The result obtained with an antiserum to the locust lysozyme is illustrated in Fig. 3. Only chicken lysozyme showed a pronounced cross-reactivity in this ELISA test; the absence of cross-reactivity with the other bird lysozymes may be due to the low titer of the antiserum to the locust lysozyme. Since the titers of the antisera to the bird lysozymes were much higher, it seemed that reciprocal tests with the locust lysozyme as antigen would be more suitable for demonstrating the presence of a weak cross-reaction.

To compare the abilities of different antisera to bird lysozymes to react with the same locust antigen, it was necessary to normalize the ELISA response curves of the different antisera with their homologous antigens. As shown in Fig. 4A, very similar curves were obtained in the homologous ELISA systems when the antisera to locust, chicken, Japanese quail, duck A, and duck B, and turkey lysozymes were diluted 1/2000, 1/4000, 1/20,000, 1/15,000, 1/15,000, and 1/30,000, respectively. The different antisera were then used at these dilutions for testing of their abilities to react with the locust lysozyme. The results illustrated in Fig. 4B demonstrate that the highest level of cross-reaction was observed with antiserum to chicken lysozyme, and then, in decreasing order, with antisera to Japanese quail, turkey, and duck lysozymes.

Discussion

The presence of lysozymelike substances has been reported in several insect orders (Malke 1965; Mohrig and Messner 1968; Hultmark et al. 1980), and some of these substances have subsequently been characterized as true lysozymes (Powning and Davidson 1976; Fernandez-Sousa et al. 1977). The

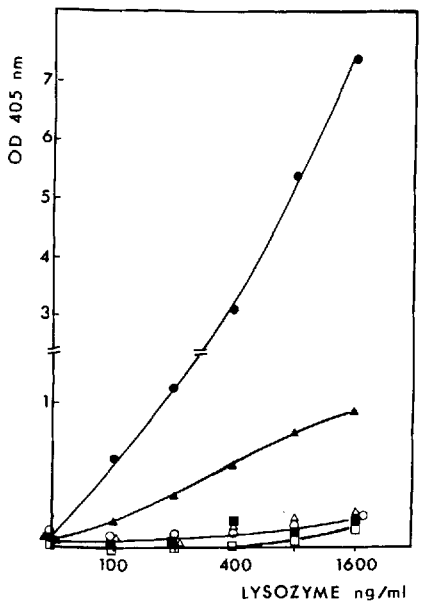

Fig. 3. Indirect ELISA with locust lysozyme antiserum. Coating was done with lysozymes from locust (●), chicken (▲), turkey (△), Japanese quail (○), duck A (■), and duck B (□). The antiserum was diluted 1/2000; goat anti-rabbit conjugate was diluted 1/2000 and incubated for 3 h at 37°C. The substrate hydrolysis was performed for 2 h at 37°C

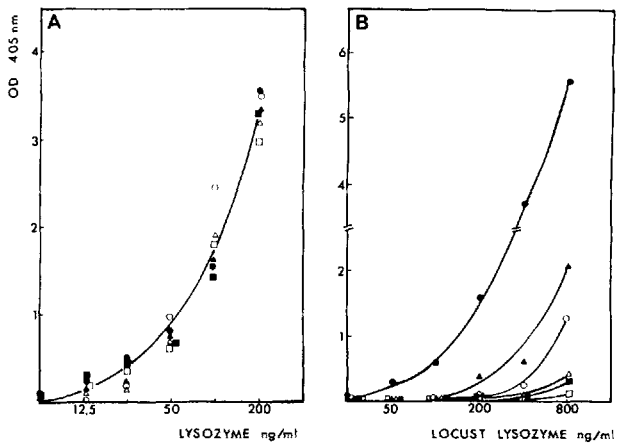

Fig. 4A,B. **A** Indirect ELISA with six homologous lysozyme–anti-lysozyme systems. The dilutions of the different lysozyme antisera were adjusted to give OD_{405} = 3.6–5 at a concentration of homologous antigen of 200 ng/ml. The lysozyme antiserum dilutions were as follows: locust, 1/2000 (●); chicken, 1/4000 (▲); Japanese quail, 1/20,000 (○); turkey, 1/30,000 (△); duck A, 1/15,000 (■); and duck B, 1/15,000 (□). Goat anti-rabbit conjugate was diluted 1/2000; the substrate hydrolysis was done for 1 h at 37°C. **B** Indirect ELISA showing the reactions of locust lysozyme with antisera against lysozymes of locust (●), chicken (▲), Japanese quail (○), turkey (△), duck A (■), and duck B (□). Coating was done with purified locust lysozyme. Antisera were used at the same dilutions as in Fig. 4A and were incubated for 2.5 h. Goat anti-rabbit conjugate was diluted 1/2000 and incubated for 3 h. Substrate hydrolysis was done for 2 h at 37°C

presence of lysozyme in invertebrates is not restricted to the phylum Arthropoda; it is found also in worms (Périn and Jollès 1973), Mollusca (Cheng and Rodrick 1974; McHenery et al. 1979), and Echinodermata (Jollès and Jollès 1975).

The aim of the present study was to establish whether immunological techniques could detect a relationship between insect and bird lysozymes. Partial sequence analysis of lysozymes of Lepidoptera indicated that bird and insect lysozymes probably differ in their sequences by about 60% (Jollès et al. 1979). Immunological comparisons of bird lysozymes by means of the microcomplement fixation and precipitin techniques have shown that serological cross-reactivity disappears when sequences of monomeric globular proteins differ by more than 30%–40% (Prager and Wilson 1971a). The existence of serological cross-reactions between the locust and bird lysozymes was therefore tested for by a more sensitive enzyme immunoassay. This indirect ELISA technique has been used successfully for detecting immunological cross-reactions between viral proteins that differ in sequence by as much as 60% (Van Regenmortel and Burckard 1980).

The results presented in Fig. 2 and Table 1 show that the degrees of cross-reactivity among five bird lysozymes as measured by ELISA agree approximately with earlier results obtained by microcomplement fixation and precipitin tests (Prager and Wilson 1971a,b). The lysozyme concentrations used in ELISA could be estimated only roughly, and the accuracy of the ELISA comparisons was therefore rather low.

The existence of immunological cross-reactivities between the locust lysozyme and the various avian lysozymes could be demonstrated by means of the indirect ELISA technique (Figs. 3 and 4). It is unlikely that the observed cross-reactions could have been due to contamination, as in the earlier report of a serological relationship between a worm and a bird lysozyme (Prager et al. 1978). The reactant concentrations were normalized in all ELISA tests and no large excess of locust enzyme was needed to reveal cross-reactivity (Figs. 3 and 4). The bird lysozymes, obtained from Dr. E.M. Prager, could not have contained any insect lysozyme. To produce a spurious cross-reaction resembling the reaction shown in Fig. 4B, the locust lysozyme would have to have been contaminated by 25% chicken lysozyme. The weak cross-reactivity observed between the locust and turkey (or duck) lysozymes also speaks against the possibility of such a massive cross-contamination of the locust enzyme preparation with chicken lysozyme. Furthermore, the difference in electrophoretic mobility between locust and chicken lysozymes ensures that a large contamination could not have remained unnoticed during the purification procedure (Zachary and Hoffmann 1984).

It is well known that distant antigenic relation-

ships are detected more easily by methods such as ELISA and radioimmunoassays, which require antibodies to react with only one site on the antigen, than by methods such as precipitin and complement fixation tests that require the antigen to be multivalent (Prager and Wilson 1971a). The indirect type of ELISA used in this work is also superior to a direct type of ELISA for demonstrating the existence of weak cross-reactions (Crook and Payne 1980; Van Regenmortel and Burckard 1980; Rybicki and Von Wechmar 1981). Although results from ELISA are less easily quantified in a reproducible manner than complement fixation tests, it is a simpler technique to use. Its ability to detect distant relationships between antigens that differ in sequence by as much as 60% should make it a useful tool in the study of protein evolution.

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