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Common antigenic properties of a g-type (goose) and a c-type (duck) egg white lysozyme: Antibody responses in rabbits and mice

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Abstract. Embden goose (GEWL) and Barbary duck (DEWL) egg white lysozymes possess different amino acid sequences corresponding to the g-type and c-type, respectively. GEWL was shown to be a better immunogen than DEWL in both rabbits and mice. The antigenicity of the two lysozymes was tested using different techniques (i.e. indirect ELISA, inhibition tests and immunoabsorption experiments). Injection of either GEWL or DEWL into rabbits and mice induced both specific antibodies and cross-reacting antibodies. Moreover, anti-GEWL antibodies, in contrast to anti-DEWL antibodies, did not cross-react with hen egg white lysozyme (HEWL), a c-type lysozyme. While the structure of GEWL was not modified after binding to plastic, DEWL was denaturated, but it did keep some native epitopes. It was concluded that g-type and c-type lysozymes, which have different amino acid sequences, exhibit strong common antigenic properties.
Key words. Bird lysozymes; immunogenicity; antigenicity; ELISA.

Lysozyme (E.C. 3.2.1.17) is an enzyme found in various tissues and secretions, which hydrolyses 1,4-β linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in the bacterial cell wall. Lysozyme has been extensively used as a model system in a large number of studies[18] of protein sequence determinations[17,18], X-ray crystallography[6,22], mechanisms of enzyme action[6], molecular evolution[18,19], and immunology[4,5]. Lysozymes have been found not only in mammals, birds, fishes and invertebrates but also in bacteriophages, bacteria, fungi and plants[18]. These lysozymes belong to different enzyme families and in many instances are quite different structurally.

The type of lysozymes found in hen egg white (c-type, molecular mass: 14.5 kD), whose sequence and properties are well-known, has been found to be present in the egg white of only two orders of birds, the Galliforms and the Anseriforms[23]. In contrast, the lysozyme which was originally found in goose egg white (g-type, molecular mass: 21 kD)[8,9] has been detected in a large number of avian orders[23]. The taxonomic distribution of g-type lysozyme is thus broader than that of c-type lysozyme.

Schoentgen et al.[24] found only slight amino acid sequence homology between g-type and c-type lysozymes, which was localized around the active site of the enzymes. However, a partial common domain structure for both types of lysozymes has been determined by crystallography[12].

The immunological properties of hen egg white lysozyme (HEWL) have been studied by many workers[3,4,16,20]. The c-type lysozyme contains several major immunologically-active regions around the disulphide bridges, and in such regions particular amino acid residues (such as Arg 68) have a stronger impact than others on the antigenic reactivity[4,15]. Anti-HEWL polyclonal serum[15,16,20] has been shown to cross-react with different c-type lysozymes (i.e. duck, quail, turkey...) which had been differentiated by monoclonal antibodies[13,25,26]. However, it has been reported that antibodies directed against the c-type HEWL do not cross-react with the g-type lysozyme, and vice versa, either in a microcomplement fixation procedure or in immunodiffusion tests[2,3].

It has been shown that when enzyme-linked immunosorbent assay (ELISA) tests are used to investigate cross-reactions between proteins, the results may vary because some proteins are denatured by binding to the plastic. In this study we were able to demonstrate a strong anti-

genic cross-reactivity between goose (g-type) and duck (c-type) lysozymes in rabbits and mice, using ELISA techniques.

Materials and methods

Hen egg white lysozyme (HEWL) was purchased from SIGMA (St. Louis, USA). Goose egg white lysozyme (GEWL) was purified by Danioux and Jollès as previously described[9].

Duck egg white lysozymes (DEWL) II and III[14] were purified together from duck eggs (*Cairina moschata* also called Barbary). Duck egg whites diluted in water at the ratio of 1/3 (v:v) were adjusted to pH 6 with 10% citric acid in the presence of 0.02% sodium azide and were agitated overnight at 4 °C (Thapon, personal communication). After filtration through Whatmann paper (2V), 50 ml of the supernatant, adjusted to pH 5.5, was further purified at room temperature by chromatography on a CMB52 (SERVA) gel column (12 ml) equilibrated in a 10 mM ammonium acetate buffer at pH 5.5. The lysozyme bound to the resin was washed with the same buffer, then eluted with 200 ml of an ammonium acetate buffer gradient from 10 mM up to 1 M at pH 5.5. The flow rate of the column was 1 ml/min leading to two peaks at 0.25 M and 0.7 M, respectively. The second peak contained 30 mg of pure DEWL, as shown by SDS-PAGE analysis (12%) in the presence of β-mercapto-ethanol, followed by silver staining[21]. A single band (at 14.5 kD) was identified by immunoblotting with a monoclonal antibody directed against HEWL which is known to cross-react with DEWL (generously donated by Dr S. Smith-Gill).

Immunization. Six-month-old, female, white New-Zealand rabbits were given an intradermal injection of 100 µg of either GEWL or DEWL in complete Freund's adjuvant (CFA), and the sera were harvested 15 days later. The rabbits received 4 intradermal boosts, each of 100 µg of antigen, in incomplete Freund's adjuvant (IFA) at monthly intervals, and sera were collected 5, 10 and 15 days after each boost. Pooled sera were stored at 4 °C after addition of 0.02% sodium azide. A similar immunization was performed on three-month-old, female, Balb/c mice with 25 µg of antigen, and sera were collected 10 days after each boost. For each antigen, 2 rabbits and 5 mice were used.

Indirect enzyme linked immunosorbent assay (indirect ELISA). The solid-phase assay using antigen bound to a solid support was an ELISA modified from that of Engvall and Perlmann[11]. All reagents were used at a volume of 100 µl/well except for the substrate, which was used at 200 µl/well. Plastic wells (maxi sorp, n° 4–42404 Nunc) were coated with 3 µg/ml of antigen (protein concentration assayed with Protein Assay Reagent [Pierce, USA]) in 0.05 M carbonate buffer at pH 9.6 for 2 h at 37 °C, or overnight at 4 °C. Coated plates were then washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20. A 5% fat-free milk solution in carbonate buffer was then added to each well and left for one hour at 37 °C. After 5 washes, the plates were incubated with either anti-GEWL or anti-DEWL serum at different dilutions in PBS, for 2 h at 37 °C or overnight at 4 °C. The plates were washed again and then incubated with an anti-species (mouse or rabbit as appropriate) IgG coupled to horse radish peroxidase (1/3000 in PBS, BIO-RAD) for one hour at 37 °C. Plates were washed 10 times before incubation with a freshly-prepared substrate solution containing 100 µl of azino-ethyl-benzthiazolin-sulphonate (stock solution 100 mg/4.5 ml water) and 20 µl of 35% hydrogen peroxide in 20 ml of 0.05 M citrate buffer at pH 4. The plates were read at 405 nm after 20 min at 37 °C. For each assay, controls for non-specific binding of anti-lysozyme antibodies and enzyme-labelled antibodies were included.

An indirect ELISA test was performed with different concentrations of antigen (37 ng/ml to 3 µg/ml) and then serum dilutions were chosen as the maximum of absorbance obtained for 3 µg/ml of homologous antigen.

ELISA inhibition tests. Inhibition was performed by pre-incubating various concentrations of either GEWL, DEWL or HEWL with the quantity of antibodies which in the indirect ELISA test exhibited 50% activity. After 2 h at 37 °C this mixture was incubated in plates coated with 3 µg/ml of GEWL or DEWL and the assay was completed as described above.

Preparation of the immunosorbent. Five ml of goose egg white (containing about 1 mg/ml of lysozyme) was fixed with 400 µl of 25% glutaraldehyde[27]. This produced a gel which was passed through a syringe with needles of decreasing diameter from 1.2 to 0.3 mm and was then saturated with 0.1 M glycine. The gel was washed in PBS until the absorbance at 280 nm was almost zero, and was then washed with a 1 M glycine-HCl buffer at pH 2.8. It was then washed in a 1 M K-dihydrogen-phosphate solution before regeneration with PBS. The gel was washed in 10 ml of each solution (PBS, glycine/HCl buffer, K H_2PO_4) containing 0.01% sodium azide then centrifuged for 30 min at 1500 × g. The entire procedure was performed at 3–5 °C.

Five mg of purified DEWL was bound to 5 ml of a preactivated gel (ACA 22, SERVA). Absorbance at 280 nm showed that 4 mg of DEWL was bound to the gel, which was introduced into a column and treated as above.

Preparation of affinity-purified antibodies. On GEWL gel: One ml of each rabbit anti-lysozyme serum was diluted ten times in PBS then mixed with the gel overnight at 4 °C under agitation. After centrifugation for 15 min at 3000 × g at 4 °C, the supernatant was harvested. The bound immunoglobulins (Ig) were eluted by 10 ml of the glycine buffer and immediately neutralised with crystallised Tris and then dialysed against cold PBS. The

serum was passed 4 times through the gel after it had been regenerated and then the antibodies were tested in an ELISA test.

On DEWL gel: One ml of each rabbit anti-lysozyme serum was applied on the DEWL column, equilibrated in PBS, and then a continuous flow (1 ml/min) was imposed overnight at 4 °C. The unbound proteins were harvested before the washing of the gel with PBS, then the Ig were eluted in glycine/HCl buffer and treated as described above. The column was regenerated with the K H_2PO_4 solution, then equilibrated in PBS.

Results

Immunogenicity of GEWL and DEWL. Rabbit and mouse sera before immunization were free of anti-lysozyme antibodies. By an indirect ELISA test, GEWL was found to induce slightly higher antibody titres to homologous antigen (1/250000) than to DEWL (1/50000) (table). In rabbit anti-GEWL serum, antibodies cross-reacting with DEWL appeared after the second boost. No antibodies bound to HEWL. In rabbit anti-DEWL serum, antibodies cross-reacting with GEWL appeared after the first boost. This serum cross-reacted strongly with HEWL. Early antisera were more specific than the hyperimmune sera.

Antigenicity of GEWL and DEWL. By indirect ELISA tests, rabbit anti-GEWL serum (fig. 1 a) cross-reacted with DEWL at 1 or 3 µl/ml but not with HEWL. Rabbit anti-DEWL serum (fig. 1 b) had a slight cross-reaction with GEWL at 1 or 3 µg/ml and had a strong cross-reaction with HEWL. For further experiments, 3 µg/ml of antigen were used.

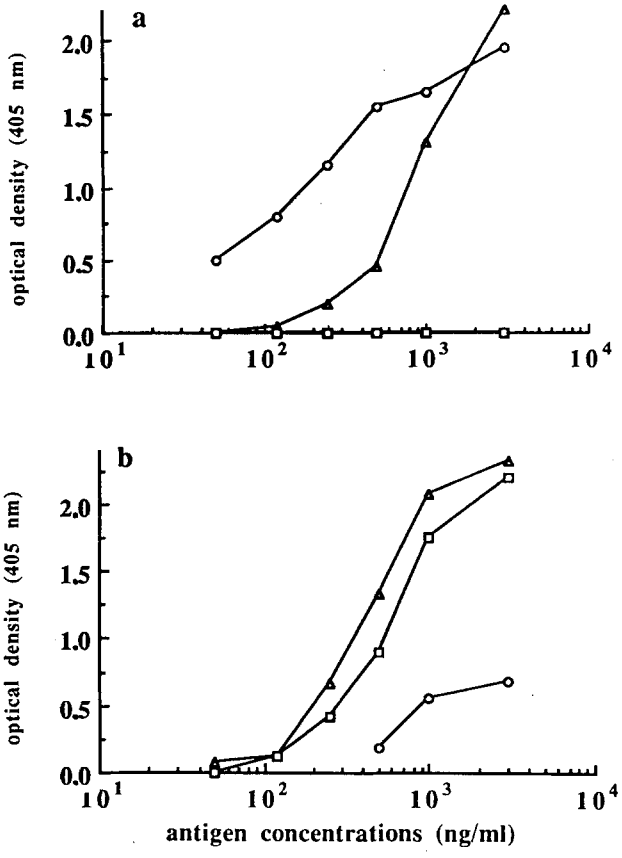

Figure 1. Antigen concentration curves. Checkerboard titrations of hyperimmune rabbit sera (*a* anti-GEWL 1/10000 and *b* anti-DEWL 1/3000) against different concentrations of lysozyme (goose: O; duck: Δ and hen: □) as tested by indirect ELISA.

Immunogenicity of goose (GEWL) and duck (DEWL) lysozymes. Immunogenic properties of four different sera were tested by indirect ELISA. Antigens were coated as a 3 µg/ml solution (goose egg white lysozyme: GEWL; duck egg white lysozyme: DEWL and hen egg white lysozyme: HEWL).
Dilutions are given for O.D. = 0.5 after subtraction of background.

Sera	Antigens coated on plastic plates		
	GEWL	DEWL	HEWL
Anti-GEWL			
Rabbit serum			
Non-immune serum	-	-	-
Primary injection	1/1000	-	-
First boost	1/3000	-	-
Second boost	1/100000	1/3000	-
Hyperimmune serum	1/250000	1/50000	-
Mouse serum			
Non-immune serum	-	-	-
Primary injection	1/3000	-	-
First boost	1/20000	-	-
Second boost	1/20000	-	-
Hyperimmune serum	1/20000	1/3000	<1/100
Anti-DEWL			
Rabbit serum			
Non-immune serum	-	-	-
Primary injection	-	1/300	1/300
First boost	1/10000	1/50000	1/50000
Hyperimmune serum	1/10000	1/50000	1/50000
Mouse serum			
Non-immune serum	-	-	-
Primary injection	1/100	1/1000	1/1000
First boost	1/400	1/4000	1/1000
Hyperimmune serum	1/1000	1/6000	1/1000

I^{125} labelled goose and duck lysozymes bound identically to the plastic plate, and antigenic properties were not modified (i.e. as detected by radioactivity measurements and by indirect ELISA; data not shown).

By inhibition test, rabbit anti-GEWL serum, as tested on GEWL coated plates, was 100% inhibited by GEWL and 25% inhibited by DEWL in solution; there was no inhibition by HEWL (fig. 2 a). Thus 100% of these antibodies bound both GEWL in solution and GEWL coated on plastic. This serum contained at least 25% antibodies cross-reacting with native DEWL. The same serum, as tested on a DEWL-coated plate, exhibited 100% inhibition with a very small amount of GEWL while the same inhibition required a larger amount of DEWL (fig. 2 b). A high concentration of HEWL was required to inhibit only 60% of anti-GEWL rabbit antibodies tested on DEWL. Thus, most antibodies seemed to be specific for GEWL and only a small proportion could bind both goose (g-type) and duck (c-type) lysozymes in solution. Similar results were obtained with mouse anti-GEWL serum but cross-reacting antibodies recognized both DEWL and HEWL (data not shown). In

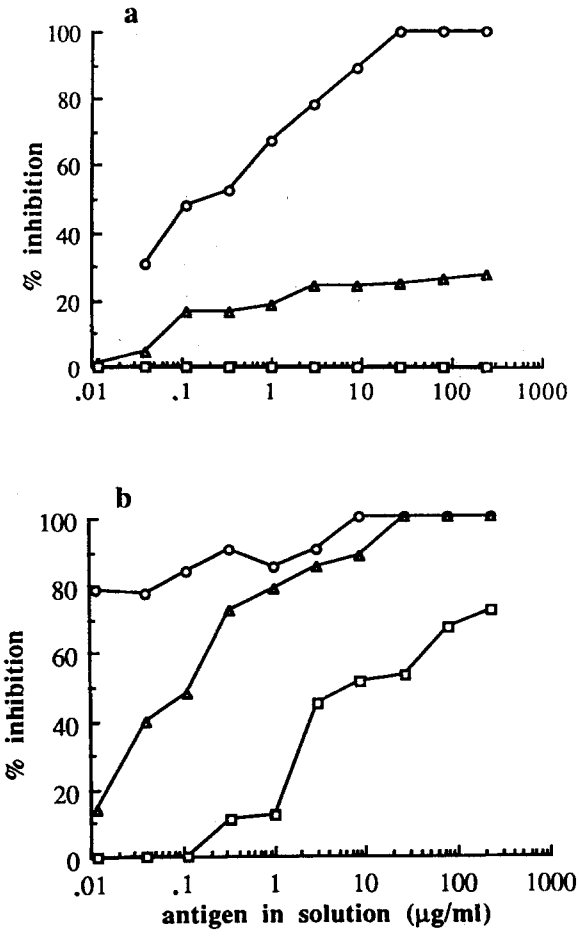

Figure 2. Specificity of rabbit anti-goose lysozyme serum as measured by inhibition tests. Percentage inhibition was checked with three lysozymes in solution (goose: O; duck: Δ and hen: □) at different concentrations as measured by indirect ELISA. Antigens were coated as a 3 µg/ml solution (*a* on goose lysozyme and *b* on duck lysozyme).

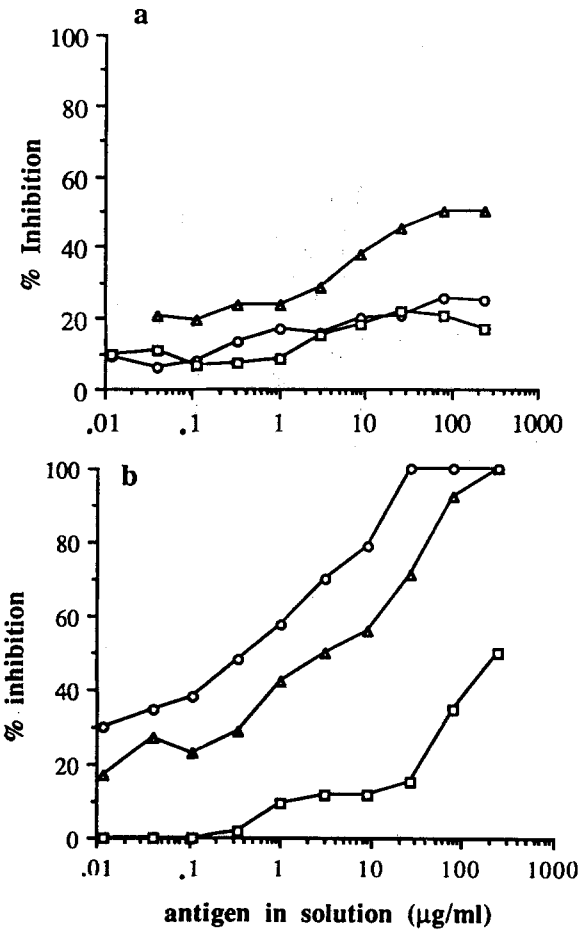

Figure 3. Specificity of rabbit anti-duck lysozyme serum as measured by inhibition tests. Percentage inhibition was checked with three lysozymes in solution (ie goose: ○; duck: △ and hen: □) at different concentrations as measured by indirect ELISA. Antigens were coated as a 3 µg/ml solution (*a* on duck lysozyme and *b* on goose lysozyme).

the rabbit serum raised against DEWL, only 50% of the antibodies which bound DEWL when coated on plastic were able to bind DEWL in solution (fig. 3a). Thus, DEWL was mostly denatured by its binding to the plastic. In addition, a population of antibodies does exist which bind DEWL only in solution and not on plastic (see below). 100% inhibition of the binding of anti-DEWL antibodies to plastic-bound GEWL was obtained with either GEWL or DEWL, but only 50% inhibition was reached with a high concentration of HEWL (fig. 3b). Thus, there were antibodies which recognized DEWL and GEWL but not HEWL. Mouse anti-DEWL serum exhibited similar properties (data not shown).

In immuno-absorption experiments, when the rabbit anti-GEWL serum applied on a homologous column (i.e. GEWL) all activity both against GEWL and against DEWL was fully absorbed (fig. 4a), as tested by indirect ELISA. Vice versa, all recovered specific immunoglobulins (Ig) (no antibody reactivity was recovered from the rinsing passing-through serum) bound both GEWL and DEWL but not HEWL in indirect ELISA (fig. 5a).

The same rabbit anti-GEWL serum applied on a heterologous column (i.e. DEWL) kept about 1/3 of its reactivity against both GEWL and DEWL (fig. 4b) Ig recovered from the DEWL column also showed a strong cross-reactivity with DEWL (fig. 5b) with a slight cross-reactivity with HEWL.

The rabbit anti-DEWL serum kept some reactivity against DEWL after immunoabsorption on the homologous column (i.e. DEWL) (fig. 4c) while antibodies binding to GEWL were fully removed. Recovered Ig were fully active against DEWL (fig. 5c) and HEWL and cross-reacted with GEWL. Thus, there are common native epitopes on goose and duck lysozymes.

The same anti-serum when absorbed on GEWL, kept about 1/3 of its reactivity on DEWL (fig. 4d), while purified Ig bound poorly (fig. 5d) on either DEWL, GEWL or HEWL.

Discussion

Up until the present time immuno-cross-reactivity has been shown among lysozymes belonging to the c-

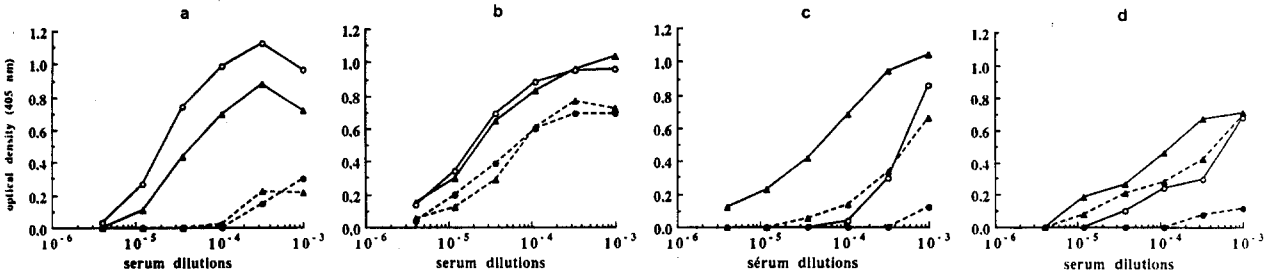

Figure 4. Specificity of rabbit anti-lysozyme sera after immuno-absorption. Rabbit anti-lysozyme sera were studied by indirect ELISA tests before (open symbols) and after immunoabsorption (closed symbols). Antigens were coated as a 3 µg/ml solution (ie goose (○) or duck (△) or hen (□) lysozyme). *a* rabbit anti-goose lysozyme serum was applied on a goose lysozyme column; *b* rabbit anti-goose lysozyme serum was applied on a duck lysozyme column; *c* rabbit anti-duck lysozyme serum was applied on a duck lysozyme column; *d* rabbit anti-duck lysozyme serum was applied on a goose lysozyme column.

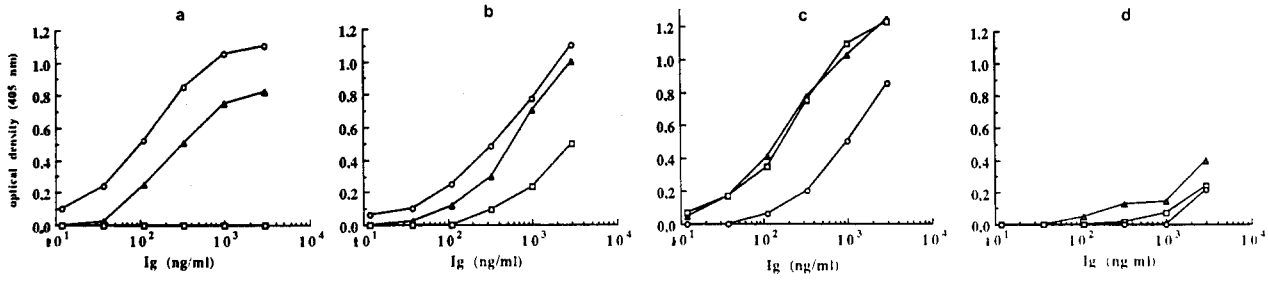

Figure 5. Specificity of immunoglobulins recovered from lysozyme columns. Indirect ELISA tests and symbols as above. *a* rabbit anti-goose lysozyme Ig recovered from a goose lysozyme column; *b* rabbit anti-goose lysozyme Ig recovered from a duck lysozyme column; *c* rabbit anti-duck lysozyme Ig recovered from a duck lysozyme column; *d* rabbit anti-duck lysozyme Ig recovered from a goose lysozyme column.

type[15,16,20], but antibodies raised against HEWL have been found not to cross-react with the g-type lysozyme, and vice versa, in either immunodiffusion tests or micro-complement fixation[3]. These results have been supported by marked differences in the amino acid sequences of the two types of lysozyme[18]. However, the three-dimensional structures of both g-type and c-type lysozymes, as studied by crystallography[12], do exhibit some similarities in certain domains.

The results of this study support the idea that common epitopes do exist on goose and duck egg white lysozymes, even though the two proteins possess many different immunogenic and antigenic properties: 1) GEWL was found to be more immunogenic than DEWL (table). This may be due to immune tolerance caused by the presence of a c-type lysozyme in mammals. 2) Rabbit anti-GEWL serum cross-reacted strongly with DEWL, whereas rabbit anti-DEWL serum cross-reacted only slightly with GEWL (figs 1a and 1b): this observation might be due to a higher affinity of anti-GEWL antibodies compared to that of anti-DEWL.

From these results, it can be concluded that epitopes present on GEWL, which were potent immunogens, were present on DEWL but absent from HEWL (figs 1a, 2a, 4a and 5a). On the other hand, antibodies from rabbit anti-DEWL serum recognized GEWL (figs 4b–d, 5b). A strong cross-reactivity of anti-DEWL sera was found with HEWL (table).

It has been shown that during protein binding to plastic, changes in the protein conformation occur, leading to a loss of some epitopes[1,7,10,28,29]. Altschuh et al.[1] observed that a viral peptide from tobacco mosaic virus did not bind monoclonal antibodies which were nevertheless able to capture this antigen in sandwich ELISA. Djavani-Ohaniance et al.[10] described one monoclonal antibody which was able to inhibit tryptophan synthase activity although it was unable to bind this enzyme in an indirect ELISA test. Ovalbumin lost 90% of its native epitopes by binding to plastic, when tested with 9 monoclonal antibodies, but these were, however, able to capture the native antigen[30]. Thus, depending upon the immunochemical test used, some epitopes may or may not be identified by the same antibodies[28].

GEWL was unaffected by binding to the plastic, as shown by a 100% inhibition of anti-GEWL antibodies by GEWL in solution (fig. 2a) and a 100% recovery of antibody activity in Ig absorbed on a GEWL column (fig. 5a). In contrast, when DEWL bound to plastic it acquired neoepitopes which were absent from the native form, as is shown by only 50% inhibition of anti-DEWL antibodies by DEWL in solution (fig. 3a), and also by immuno-absorption (fig. 4c). Ig specific for the native form of DEWL (fig. 5c) identified DEWL on plastic, showing that some native epitopes were still present.

The results reported by Arnheim and Steller[3], showing that no immunological cross-reaction occurred between

the two types of lysozyme, might be due either to the test used being insensitive or to the immunization procedures they employed, which was different from ours (they immunized animals with HEWL instead of DEWL). The use of monoclonal antibodies may resolve this point.
The work reported in the present paper shows that some common epitopes do exist on GEWL and DEWL. The cross-reactivity between the two lysozymes is likely to be due to conformational similarities[28], since only a slight sequence homology has been found on these molecules[24]. The existence of conformational similarities is supported by crystallographic observations showing some common domain structures between g-type and c-type lysozymes, despite the fact that the amino acid sequences are almost entirely different.

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Variation in regulation of aflatoxin biosynthesis among isolates of *Aspergillus flavus*

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Abstract. Two new phenotypes of *Aspergillus flavus* which exhibit novel patterns of aflatoxin production have been identified and characterized. In one of the new variants of *A. flavus*, aflatoxin is made in the absence of carbohydrate and concomitantly with growth, without a lag period. A second variant did not produce aflatoxin in the presence or absence of carbohydrate. Chemical mutagenesis of this nonaflatoxigenic strain resulted in mutant strains which produced aflatoxin on carbohydrate-containing media. The aflatoxin production pattern observed in these mutants resembled the typical production scheme, with a lag period through log phase growth.
Key words. Aflatoxin; *Aspergillus flavus*; *Aspergillus parasiticus*; secondary metabolism; genetic regulation; nutritional regulation.

The aflatoxins are a group of polyketide secondary metabolites produced by the filamentous fungi *Aspergillus flavus* and *A. parasiticus*. These compounds are toxic and carcinogenic and frequently contaminate food and feed commodities. Previous investigations on the regulation of aflatoxin production have focused on nutritional factors that enhanced or suppressed aflatoxin biosynthesis[1–4]. Media containing certain carbohy-