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OVOMUCOID AND OVOINHIBITOR ISOLATED FROM CHICKEN EGG WHITE ARE IMMUNOLOGICALLY CROSS-REACTIVE

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SUMMARY: Ovomucoid and ovoinhibitor were isolated from chicken egg white. Enzyme-linked immunosorbent assay (ELISA) and affinity chromatography of ovoinhibitor-coupled Sepharose 4B showed that about 25% of rabbit anti-ovomucoid antibody reacted with ovoinhibitor. Ovoinhibitor required about 1000 times the concentration of ovomucoid to give 50% inhibition of anti-ovomucoid antibody binding to ovoinhibitor in the plate-binding ELISA. These results suggested that ovoinhibitor possesses antigenic determinants which are conformationally homologous with those of ovomucoid and cross-react with anti-ovomucoid antibodies.

Ovomucoid is a protein (Mw: 28000) containing about 20-25% carbohydrate, and it accounts for about 10% of avian-egg white protein (1-3). Lineweaver and Murray (4) demonstrated that chicken ovomucoid is responsible for most of the trypsin inhibitory activity of chicken egg white, and Rhodes et al. (1) found that chicken ovomucoid binds only one trypsin molecule and no other enzyme molecules.

Ovoinhibitor, another protease inhibitor in egg white, was first isolated by Matsushima (5). Chicken ovoinhibitor (Mw: 49000) contains much less carbohydrate than ovomucoid, and accounts for only 1.5% of egg white protein (6,7). Capable of inhibiting simultaneously 2 mol of trypsin and 2 mol of chymotrypsin per mol of inhibitor (8), ovoinhibitor

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Abbreviations: PBS, 0.15 M NaCl/0.01 M phosphate buffer, pH 7.2; PBS-Tween, 0.05% Tween 20/PBS; BSA, bovine serum albumin; IgG, immunoglobulin class G; PVP, polyvinylpyrrolidone (Mw: 40000).

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also inhibits other proteolytic enzymes such as subtilisin and elastase (9,10).

Both ovomucoid and ovoinhibitor have been classified into Kazal type of protease inhibitors on the basis of the topological structure obtained from their amino acid sequence (11).

In the present study, the reactivity of rabbit anti-ovomucoid antibody with ovoinhibitor was investigated, and the findings showed that ovomucoid and ovoinhibitor are immunologically cross-reactive.

MATERIALS AND METHODS

Isolation of ovomucoid and ovoinhibitor: Ovomucoid was isolated from egg white of White Leghorn hens according to the procedure of Waheed and Salahuddin (12). Ovomucoid preparation was passed through a column of TLCK-chymotrypsin-coupled Sepharose 4B to eliminate ovoinhibitor as a trace contaminant in the preparation. Ovoinhibitor also was isolated from the egg white according to Matsushima (5), and further purified by affinity chromatography on a column of TLCK-chymotrypsin-coupled Sepharose 4B (13).

Preparation of anti-ovomucoid antibody: Rabbit antiserum to ovomucoid was prepared as described previously (14), and IgG fraction was prepared by passing the serum through a column of DEAE-cellulose (Whatman, DE 52) equilibrated with 10 mM phosphate buffer, pH 7.2. Anti-ovomucoid antibody was purified from the IgG fraction by affinity chromatography on a column of ovomucoid-coupled Sepharose 4B.

Enzyme-linked immunosorbent assay (ELISA): The binding of anti-ovomucoid antibody to ovoinhibitor was examined by a plate-binding ELISA (15). Flat bottomed microtiter plates (Coster) were coated overnight at 4°C with 150 µl protein solution (1 µg/ml) in 0.1 M carbonate buffer, pH 9.5, and washed with three changes of PBS-Tween. One hundred and fifty µl of antibody solution appropriately diluted with PBS-Tween containing 2% PVP and 0.2% BSA was added to each well and incubated 120 min at 37°C. The plates were washed with PBS-Tween, and 150 µl of peroxidase-coupled goat anti-rabbit IgG (Cappel Laboratories) appropriately diluted with PBS-Tween containing 2% PVP and 0.2% BSA, was added and incubated 120 min at 37°C. After washing with five changes of PBS-Tween, 150 µl of substrate (0.4% o-phenylene-diamine, 0.003% H_2O_2, in 0.1 M citrate-phosphate buffer, pH 5.0) was added and plates were incubated 15 min at 25°C. The amount of peroxidase product in each well was quantitated spectrophotometrically at a wavelength of 492 nm. For competitive inhibition assay, 100 µl of competitor protein and 50 µl of antibody solution were added to the wells coated with ovoinhibitor. The plates were incubated overnight at 37°C and processed as above.

Preparation of insolubilized proteins: TLCK-chymotrypsin (SIGMA Chemical Company), ovomucoid and ovoinhibitor were

insolubilized by incubating with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) as described previously (14).

Determination of proteins: The protein concentrations were determined by the optical absorption at 280 nm using E_{280}= 14.0, 4.6 and 7.2 for antibody, ovomucoid and ovoinhibitor, respectively.

Immunodiffusion: Ouchterlony immunodiffusion (16) was performed in 1.0% agarose in PBS containing 0.1% NaN_3 (14).

RESULTS

The immunodiffusion of ovomucoid and ovoinhibitor against anti-ovomucoid antibody was shown in Fig. 1. A clear and single precipitin arc was produced between ovomucoid and anti-ovomucoid antibody. Ovoinhibitor also formed only a trace amount of precipitin arc with anti-ovomucoid antibody.

Figure 2 shows the reactivity of anti-ovomucoid anibody with ovomucoid and ovoinhibitor as measured by ELISA. Anti-ovomucoid antibody bound not only to ovomucoid but also to ovoinhibitor, though less antibody reacted with ovoinhibitor than with ovomucoid at the same antibody concentration. The antibody did not bind to the well coated with BSA as control. Hence, the non-specific interaction between antibody and ELISA plates was negligible in this experimental condition.

The antibodies which react with ovoinhibitor were fractionated from anti-ovomucoid antibody by affinity chromato-

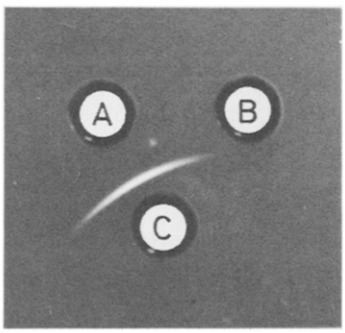

Figure 1: Immunodiffusion of anti-ovomucoid antibody against ovomucoid and ovoinhibitor.
A: ovomucoid; B: ovoinhibitor; C: anti-ovomucoid antibody.

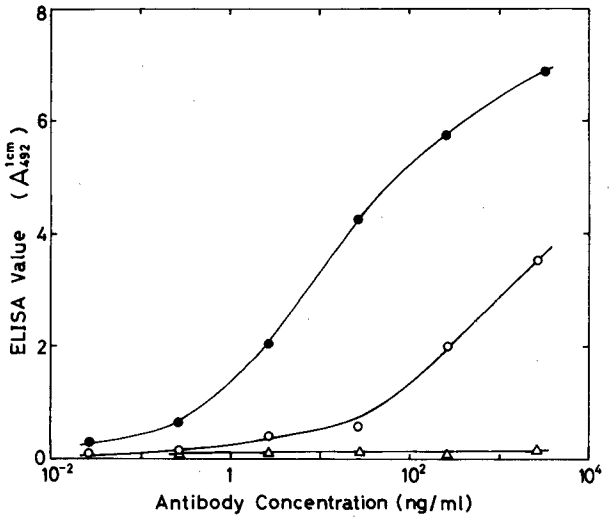

Figure 2: Reactivity of anti-ovomucoid antibody with ovomucoid and ovoinhibitor as measured by ELISA.
ELISA plates were coated with ovomucoid (—●—), ovoinhibitor (—○—) and BSA (—△—). The antibody amount which bound to the plates was expressed as peroxidase activity. Each point represents the means of 3 determinations.

graphy on a column of ovoinhibitor-conjugated Sepharose 4B.

Figure 3 shows the elution profile of the antibody from the

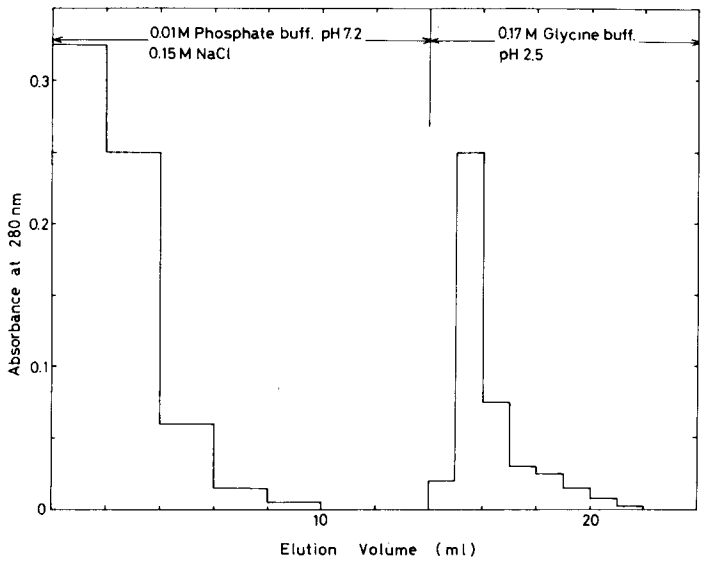

Figure 3: Affinity chromatography of anti-ovomucoid antibody on a column of ovoinhibitor-coupled Sepharose 4B.
Anti-ovomucoid antibody was added to the resin suspended in PBS and incubated 120 min at 37°C. The resin was packed in a column and eluted with PBS until the absorbance at 280 nm of eluate reached zero. The adosorbed antibody was recovered by elution with 0.17 M glycine/HCl buffer, pH 2.5.

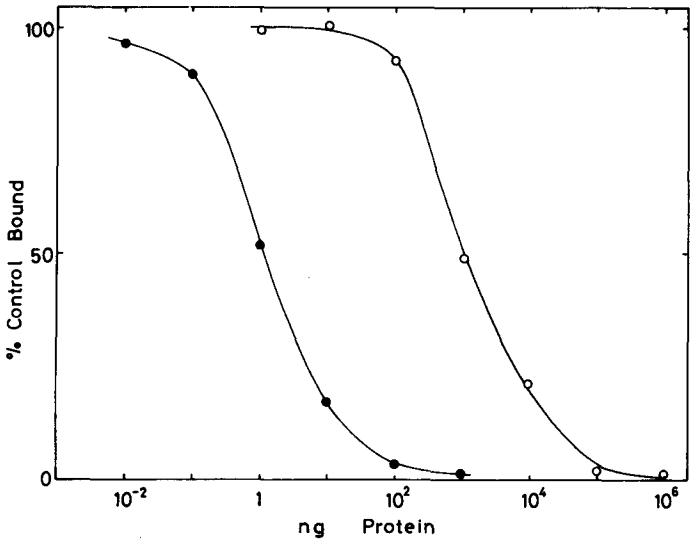

Figure 4: Competitive inhibition of the binding of anti-ovomucoid antibody to ovoinhibitor.
The proteins used as competitive inhibitor were ovomucoid (—●—) and ovoinhibitor (—o—). Each point represents the means of 3 determinations.

column. The antibody capable of binding to ovoinhibitor was calculated to be about 25% of the total anti-ovomucoid antibody by measuring the peak areas in the elution pattern. The fraction eluted with PBS no longer contained any antibody with reactivity to ovoinhibitor.

Figure 4 shows the competitive inhibition of the binding of anti-ovomucoid antibody to ovoinhibitor. Both ovomucoid and ovoinhibitor inhibited binding of anti-ovomucoid antibody to the plate-binding ovoinhibitor. However, ovoinhibitor required about 1000 times the concentration of ovomucoid to give 50% inhibition of antibody binding to ovoinhibitor in the ELISA plate-binding assay.

DISCUSSION

Any discussion of an immunological cross-reaction between ovomucoid and ovoinhibitor must deal with the important problem

of possible ovomucoid and ovoinhibitor preparation contamination of each other, because the two proteins are prepared from chicken egg white of the same origin. In the present experiment, affinity chromatography on a column of TLCK-chymotrypsin-coupled Sepharose 4B was an effective technique for the protein isolation, because ovoinhibitor inhibits chymotrypsin but ovomucoid does not. In fact, the ovomucoid preparation showed no chymotrypsin inhibitory activity. Thus, ovomucoid and ovoinhibitor preparations would be pure enough to use in the immunochemical experiments.

The reactivity of anti-ovomucoid antibody with ovoinhibitor was first found by gel diffusion (Fig. 1). The precipitin bands suggested that only a part of anti-ovomucoid antibody reacted with ovoinhibitor and/or that the affinity between the antibody and ovoinhibitor was rather weak. In any case, gel diffusion analyses are of a qualitative nature and not always easy to interpret. Hence, enzyme immunoassays, a more sophisticated technique, were carried out.

The plate-binding ELISA (Fig. 2) and the immunoaffinity chromatography (Fig. 3) provided sufficient evidence to demonstrate that some anti-ovomucoid antibodies reacted with ovoinhibitor. Moreover, the competitive inhibition assay (Fig. 4) indicated that the antibodies with reactivity to both proteins had a weaker affinity to ovoinhibitor than to ovomucoid. Ovoinhibitor would possess antigenic determinants which are conformationally homologous with those of ovomucoid and cross-react with anti-ovomucoid antibodies.

It is presumed that multiheaded protease inhibitors originally consist of only one domain and are converted into a two-domain protein and then into a protein of three domains or more by gene elongation resulting from unequal crossing-over

(17). Ovomucoid and ovoinhibitor consist of 3 and 6 domains, respectively, which are topologically homologous (11). These two protease inhibitors in chicken egg white might originate from the same single-domain proteins.

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