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Antigenic Properties of Ovalbumin Following Heat Denaturation at Different Temperatures: Comparison with Enzymatic Denaturation

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Eighteen monoclonal antibodies (MAbs) raised from mice which were immunized with either native ovalbumin (NOA) or ovalbumin which had been heat denatured at 100°C, (HDOA) were used to study antigenic modifications induced by either heat or subtilisin treatment. Using enzyme immunoassays (EIA) we have defined three major groups of antigenic sites: Group I thermolabile native epitopes; Group II relatively thermostable native epitopes; Group III epitopes specific to heat-denatured ovalbumin. Heat-denatured ovalbumin can be separated into monomers and polymers which constitute 1% and 99% of the molecules respectively. Whereas MAbs belonging to Groups I and II bound to the monomeric form (mHDOA), MAbs belonging to Group III only bound to the polymeric form (pHDOA). Plakalbumin (PK) behaved similarly to pHDOA since it was recognized by many MAbs from Group III and a few from Group II. Nevertheless, PK remained as a monomeric molecule, whereas heat-denatured ovalbumin existed mainly as polymeric aggregates. The epitopes present on ovalbumin after different heating procedures (time/temperature) were found to be stable after cooling, thus allowing specific recognition by different MAbs. The critical modification of the protein structure was found to take place at 75°C. Two main conclusions can be drawn from these results: (i) heat and enzymatic denaturation of ovalbumin led to similar antigenic modifications—these may be explained by the exposure of hydrophobic residues in both HDOA and PK as evidenced from 8-anilino-1-sulphonic acid fluorescence spectra; (ii) the panel of ovalbumin-specific MAbs was able to differentiate between the various heat treatments which had been applied to ovalbumin within the range 65–85°C, even after subsequent cooling. The aggregation of ovalbumin molecules during the heating process constitutes the main type of antigenic modification.

Keywords: Ovalbumin, plakalbumin, antigen, heat denaturation, proteolysis, monoclonal antibodies, ELISA

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INTRODUCTION

Native hen ovalbumin (NOA) is a glycoprotein with a molecular weight of 43 kDa including a carbohydrate moiety which represents 3.2% of the total weight. The complete amino acid sequence has been described, and consists of a polypeptide chain of 385 amino acids with a single intra-chain disulphide bond (Nisbet *et al.*, 1981).

Subtilisin cleaves ovalbumin under suitable conditions creating a new crystalline form, plakalbumin (PK) and releasing both a heptapeptide and a small fragment corresponding to the C-terminal portion of the molecule (Thompson *et al.*, 1971). During heat treatment, intermolecular interactions determine the functional characteristics of proteins such as their gelation and foaming properties (Egelandstal, 1980, 1986; Kato & Takagi, 1987). At the molecular level it has been found that two types of modifications occur: (i) coagulation and aggregation (Hegg *et al.*, 1979; Arnfield *et al.*, 1989; Kitabatake *et al.*, 1981; Kato *et al.*, 1990) and (ii) Maillard reaction of saccharides with the protein moiety (Kato *et al.*, 1981, 1985, 1986, 1989; Matsuda *et al.*, 1985). The glycosylation of a protein has been found to play an important role by protecting the polypeptide moiety from heat denaturation (Gu *et al.*, 1989).

Ovalbumin has been widely used as a human food source and as an experimental model of hypersensitivity and oral tolerance (Stokes *et al.*, 1983; Iwata *et al.*, 1987). Antigenic and allergenic determinants on NOA have been identified and a synthetic decapeptide has been shown to encompass an Ig-binding haptenic epitope (Elsayed *et al.*, 1986, 1988). Furthermore, it is clear that heat denaturation can modify the antigenic and allergenic properties of NOA (Kato *et al.*, 1985; Matsuda *et al.*, 1985; Elsayed *et al.*, 1986). In the present study, monoclonal antibodies (MAbs) were used to demonstrate that antigenic modifications of NOA produced by heating mimic the antigenic properties of PK. This mimicry may be due to the exposure of hydrophobic regions which appear after either thermal or subtilisin treatment. Moreover, the antigenic modifications induced by different heating procedures can be identified by different MAbs.

MATERIALS AND METHODS

Chemicals

Potassium phosphate-buffered saline (PBS) and PBS including 0.1% Tween 20, v/v, and 0.5% gelatin, w/v (PBSTG) were used as buffers. Peroxidase-labelled goat anti-mouse Ig and peroxidase-labelled goat anti-rabbit Ig were from BioRad. 2,2'-azimodi-(3 ethyl-benzthiazoline-6-sulphonate) (ABTS) and 8-anilino-1-sulphonic acid (ANS) were from Sigma (St Louis, MO). The standard BioRad protein assay reagents were from Richmond, CA.

Antigens

Native ovalbumin fraction VII (NOA) was from Sigma (St Louis, MO) was prepared as described by Smith (1968) and the small fragment of PK was eliminated by gel filtration; the purity of the large PK peptide was checked by SDS-PAGE analysis. Heat-denatured ovalbumin (HDOA) was prepared by heating 10 µg per ml NOA in pH 7.5 PBS at different temperatures (50, 65, 70, 75, 80, 85 or 100°C) for 10 min; in another set of experiments the solution was heated at either 75°C or 80°C for 1, 3, 10 or 30 min. Monomeric and polymeric heat-denatured ovalbumin (mHDOA and pHDOA respectively) were prepared by heating NOA (0.5 mg ml⁻¹ in pH 7.5 PBS) for 30 min at 100°C (no coagulum was formed). Gel filtration on HPLC-TSK G 4000 SW (600×7.5 mm) resulted in two peaks at 9.5' and 20.8' for pHDOA and mHDOA respectively.

Antibodies

The production and characterization of the MAbs has been described elsewhere (Varshney *et al.*, 1990). MAbs were produced in mice in the form of ascitic fluids and were precipitated by ammonium sulphate at either 40% for IgG or 50% for IgM. The pellets were resuspended in bidistilled water and extensively dialysed against PBS. Protein estimation was performed using the standard BioRad protein assay. Many of the MAbs were of the IgG isotype (IA2A2, NA1E5, AD6C4, KD1D3, KA4B4, QA6B5, MB4D8) whereas the others were all IgM.

Rabbit polyclonal anti-ovalbumin NAc 2 was prepared by affinity chromatography (Breton *et al.*, 1989).

Enzyme Immunoassays

(1) *Direct assay.* Antigen-coated plates ($1 \mu\text{g ml}^{-1}$ in 0.1 M carbonate/bicarbonate buffer, pH 9.6) were saturated with PBSTG buffer. After washing they were incubated with MAbs at different concentrations for 1 h at 37°C, then the plates were washed again and incubated with peroxidase-labelled goat anti-mouse Ig (BioRad) for 1 h at 37°C. The enzyme activity was developed with ABTS and the optical density was measured in a Titertek Microtitration plate reader (Lab Systems, NC).

(2) *Sandwich assay.* MAb-coated plates ($5 \mu\text{g ml}^{-1}$) were incubated with different concentrations of antigen in PBSTG for 1 h at 37°C. After washing, they were incubated with rabbit anti-ovalbumin NAc2 antibodies (Breton *et al.*, 1989) and the reaction was completed as described above using peroxidase-labelled goat anti-rabbit Ig (BioRad).

(3) *Competitive assay.* The 50% fixation point of each MAb bound to antigen-coated plates was determined by a direct assay as above. MAbs were incubated at this concentration in solutions with decreasing concentrations of either pHOAD or PK for 1 h at 37°C. The mixture was then allowed to react for 1 h at 37°C with antigen-coated plates. After washing, MAbs bound to antigen-coated plates were quantified as in the direct assay.

Fluorescence assay. The fluorescence spectra were measured in 10 mm cuvettes at 22°C in a Jasco FP770 spectrofluorimeter fitted with a circulating water bath. The protein concentrations of NOA, HDOA, and PK were $10 \mu\text{g ml}^{-1}$ in PBS. The solutions were excited at 280 nm for the determination of intrinsic tryptophan fluorescence and for ANS-treated proteins, the excitation maximum was 375 nm. A stock solution of 10 mM ANS in ethanol was used and the final concentration of ethanol in each protein solution did not exceed 0.03%.

RESULTS

Binding of MAbs to NOA and PK

In a sandwich enzyme immunoassay (EIA) with MAbs as captors for the antigens and rabbit NAc2 polyclonal antibodies to reveal immunocapture of antigen, only six out of 18 MAbs exhibited a good specificity for NOA (IA2A2, NA1E5, KD1D3, KA4B4, QA6B5 and MB4D8) (Figure 1). In contrast, most of the other MAbs (10 out of 18) were specific for PK. However, two MAbs bound both antigens: IB4E5 bound PK even at low dose (80 ng ml^{-1}) and NOA at high dose ($2 \mu\text{g ml}^{-1}$), whereas the reverse was true for AD6C4 which bound NOA and PK at 400 ng ml^{-1} and $80 \mu\text{g ml}^{-1}$ respectively.

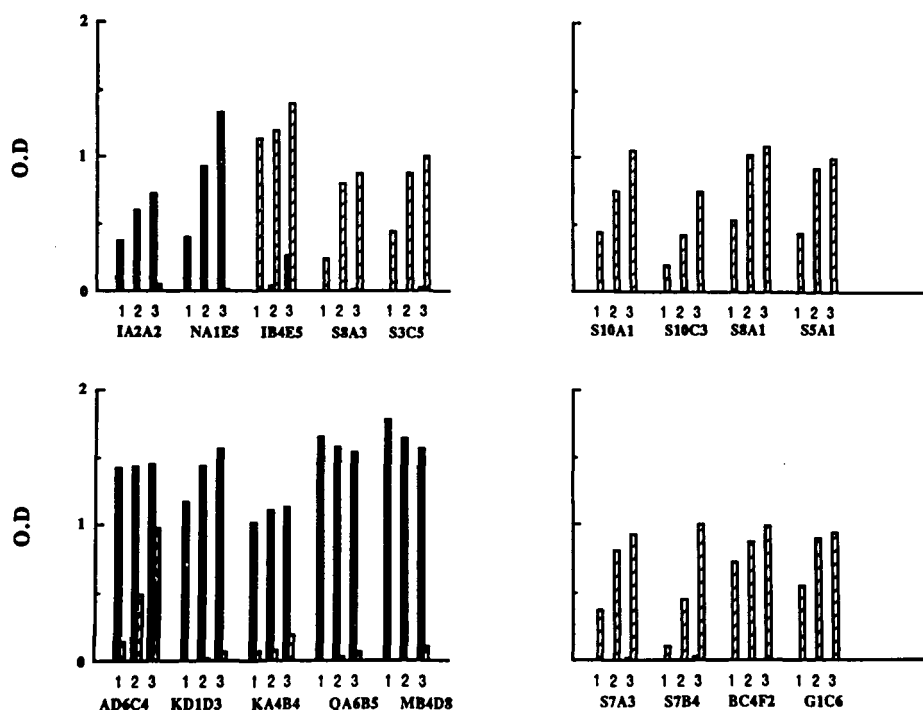


FIG. 1. Binding of native ovalbumin (NOA) and plakalbumin (PK) to different monoclonal antibodies (MAbs). Binding was evaluated by a sandwich EIA using plates coated with MAb at $5 \mu\text{g ml}^{-1}$, which was allowed to react with NOA or PK in solution at different concentration 0.08, 0.4 and $2 \mu\text{g ml}^{-1}$ (1.2.3) respectively; then with rabbit anti-OA (NAc2) antibodies and goat anti-rabbit antibodies labelled with peroxidase. Each experimental point shown in the figure is an average of at least three experiments in duplicate. (■) NOA; (▨) PK.

Binding of MAbs to mHDOA and pHDOA

After heating for 30 min at 100°C the ovalbumin solution contained about 1% monomeric and 99% polymeric forms by HPLC analysis. This demonstrated two peaks of 43 kDa and 18×10^3 kDa respectively (result not shown). Only three MAbs (IA2A2, NA1E5 and KD1D3) were specific for mHDOA and eight MAbs were specific for pHDOA (S8A3, S3C5, S10A1, S10C3, S8A1, S5A1, S7A3 and G1C6) whereas all other MAbs were able to recognize both mHDOA and pHDOA (Figure 2). Thus, three antigenic groups can be defined (Figures 1 and 2).

Group I: thermolabile epitopes identified by IA2A2, NA1E5 and KD1D3 which recognized NOA and mHDOA.

Group II: relatively thermostable epitopes identified by IB4E5, AD6C4, KA4B4, QA6B5, MB4D8, S7B4 and BC4F2.

Group III: heat-denatured epitopes identified by S8A3, S3C5, S10A1, S10C3, S8A1, S5A1, S7A3 and G1C6 which bound only to pHDOA and PK.

Antigenic Similarities between pHDOA and PK

By comparing Figures 1 and 2 it can be seen that out of 15 MAbs which captured pHDOA, three did not recognize PK (KA4B4, QA6B5 and MB4D8) whereas two

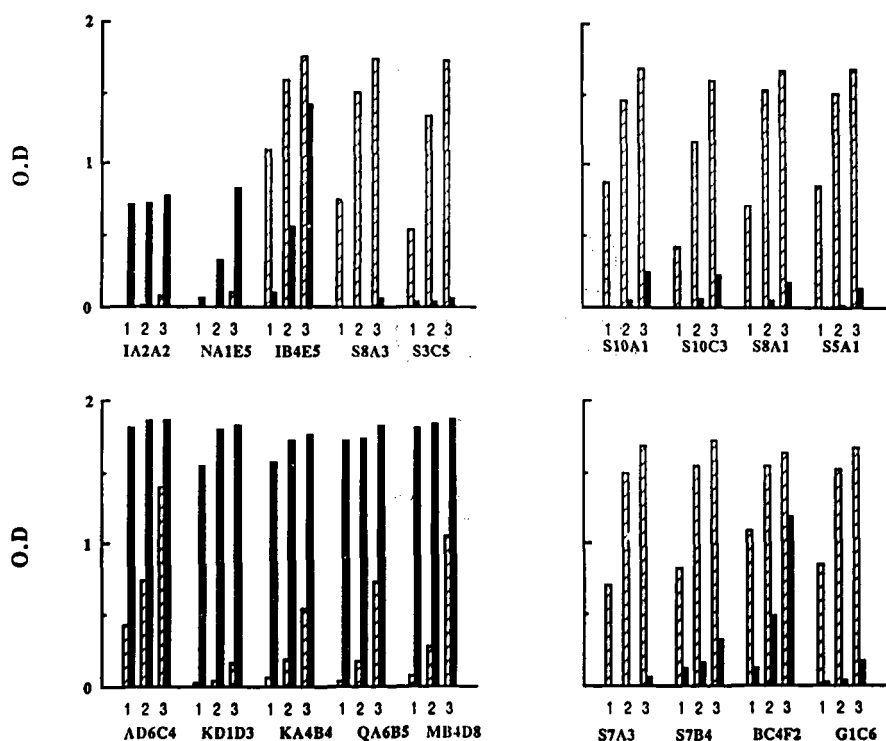


FIG. 2. Binding of monomeric heat denatured ovalbumin (mHDOA) and polymeric heat denatured ovalbumin (pHDOA) to different monoclonal antibodies. Binding was evaluated by the sandwich EIA using plates coated with MAb as in Figure 1. (▨) pHDOA; (■) mHDOA.

MAbs (S7B4 and BC4F2) not only bound pHDOA and PK but also mHDOA. Specific MAbs for NOA (Figure 1) also bound mHDOA (Figure 2). The results obtained for PK and pHDOA by immunocapture were identical to those found by the direct EIA (results not shown). In order to investigate more thoroughly the cross-reaction between pHDOA and PK, a competitive EIA was used. The results are given in Figure 3 and show that IB4E5 exhibited the same inhibition properties against PK as for pHDOA (no inhibition was detected by NOA); conversely, G1C6 was more specific for pHDOA than for PK as the amount of antigen needed to induce 50% inhibition was 30/50 times greater for PK than for pHDOA.

Changes in HDOA and PK Structures Measured by the Fluorescence Method

Tryptophan fluorescence. Native ovalbumin shows a $\lambda_{\max}$ for emission at 335 nm (Figure 4A). Heating a $10 \mu\text{g ml}^{-1}$ protein solution at 100°C for 10 min and then cooling it to room temperature did not produce any detectable shift in the fluorescence maximum, although the intensity was reduced in comparison with NOA at the same protein concentration. Likewise, PK showed reduced fluorescence intensity comparable with that of HDOA, although no apparent shift in the fluorescence maximum from the native protein was observed.

ANS fluorescence. ANS in PBS shows a fluorescence maximum at 534 nm when excited at 375 nm (result not shown). The addition of ANS to NOA did not show any change either in the fluorescence maximum nor in the shape of the fluorescence

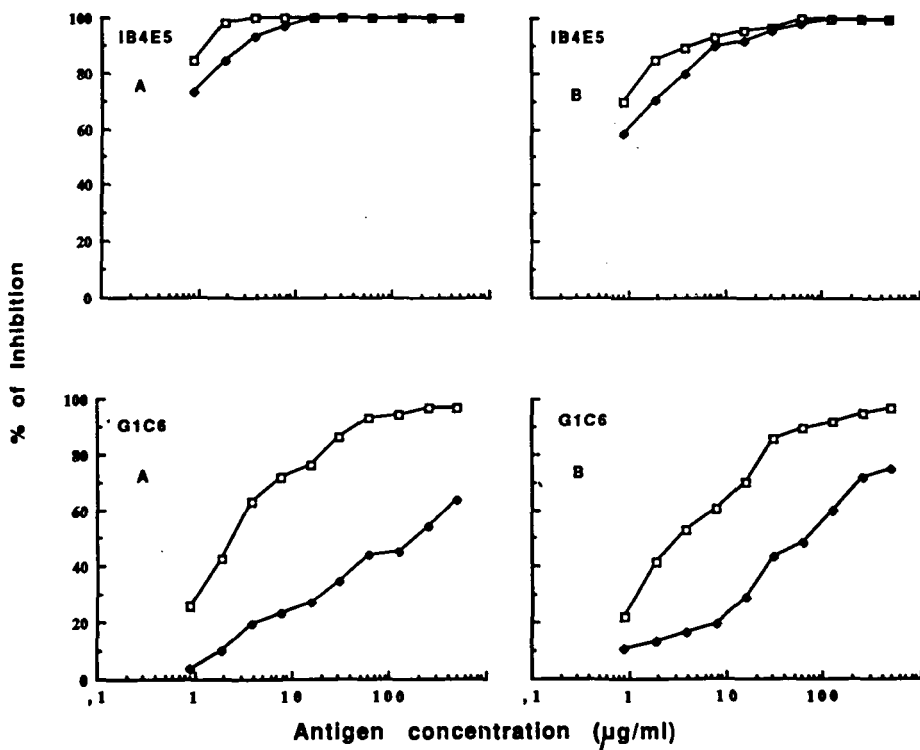


FIG. 3. Inhibition of binding of two monoclonal anti-ovalbumin antibodies to heat-denatured ovalbumin HDOA (A) or to plakalbumin PK (B) by either HDOA (□), or PK (●). The concentration of antibodies corresponding to the 50% point of direct activity were incubated with an increasing amount of antigen, that allowed to react with antigen-coated plates. Binding was normalized to 100% in the absence of inhibitor.

spectrum and the two spectra were virtually superimposable, thus confirming the observation made by Klausner *et al.* (1982). Figure 4B shows the fluorescence spectra of ANS in NOA, HDOA and PK. Both the HDOA and PK spectra showed an increased fluorescence intensity and a shift towards the blue end as compared with ANS in the presence of NOA. The fluorescence maxima is more blue-shifted and had greater intensity in the presence of HDOA. Since free (non-interacting) ANS molecules would have been present in the protein solutions, the fluorescence spectra of ANS in either HDOA or PK were obtained by subtracting the ANS-PBS spectrum from the ANS-protein spectrum (Figure 4C). As can be seen, both fluorescence spectra have maxima around 480 nm indicating that the fluorophore is in a hydrophobic environment (Klausner *et al.*, 1982). However, the fluorescence intensity of ANS in PK is much reduced compared with that for HDOA.

Identification by MAbs of the Temperature/Time of Ovalbumin of Heat Treatment

In order to determine if MAbs were able to discriminate between the structural protein modifications induced by different temperature and times of treatment, solutions of ovalbumin ($10 \mu\text{g ml}^{-1}$) were heated (10 min at 65, 70, 75, 80 or 85°C) and the reactivity of four different MAbs (KD1D3, MB4D8, QA6B5 and IB4E5) against these solutions was tested by immunocapture. KD1D3 was able to bind NOA and HDOA which had been heated up to 75°C, and showed a capacity to discriminate

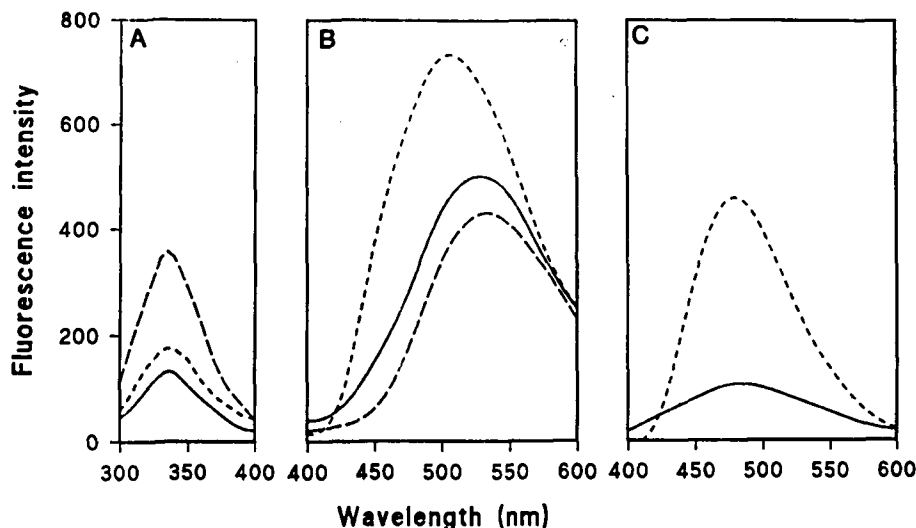


FIG. 4. (A) Intrinsic tryptophan fluorescence spectrum of NOA (—), HDOA (---) and PK (---). Protein concentrations were $10 \mu\text{g ml}^{-1}$ in PBS. The solutions were excited at 280 nm and at a temperature of 23°C . (B) ANS fluorescence in different ovalbumin solution. $10 \mu\text{M}$ of ANS was added to NOA (—), HDOA (---) and PK (---). Excitation wavelength 375 nm. (C) Difference ANS fluorescence spectra in HDOA (—) and PK (---) solutions. The samples of ANS in HDOA and PK used in (B) were measured against an ANS sample in NOA. Experimental conditions are the same as in (B).

between the 70°C and 75°C treatments and between the 75°C and 80°C treatment (Figure 5). MB4D8 and QA6B5 were able to bind NOA as well as HDOA which had been heated at 65, 70 or 75°C , and were able to discriminate between 75 and 80°C treatments and between 80 and 85°C treatments. In contrast, IB4E5 recognized HDOA heated either at 75, 80 or 85°C but completely failed to recognize NOA or HDOA heated at 65 or 70°C .

In other experiments, the length of the heat treatment necessary to modify the ovalbumin structure at different temperatures was evaluated. Solutions of ovalbumin ($10 \mu\text{g ml}^{-1}$) were heated for 1, 3, 9 or 30 min at either 75 or 80°C . That the binding of QA6B5 to HDOA at either 75 or 80°C for different times was dependent on the antigen concentration (Figure 6): at 75°C with 3 ng of antigen the binding decreased slowly from 0 to 30 min while with 100 ng of antigen no modification was detected even when OA has been heated for 30 min (Figure 5A). In contrast, when HDOA was heated at 80°C the binding of 3 ng of antigen had completely disappeared after one minute, while with 100 ng, the decrease in binding was 50% after 1 min and 100% after 30 min (Figure 5B).

It is worthwhile stressing that the concentration of ovalbumin in the test was critical and we found that for each MAb the maximum sensitivity to discriminate temperature and time of heating was different.

DISCUSSION

In a previous study (Varshney *et al.*, 1990), it was shown that MAbs were able to distinguish between NOA and 100°C HDOA. Here we show that similar epitopes can characterize ovalbumin denatured by either subtilisin or heat treatments and that such changes are supported by the appearance of hydrophobic sequences measured by

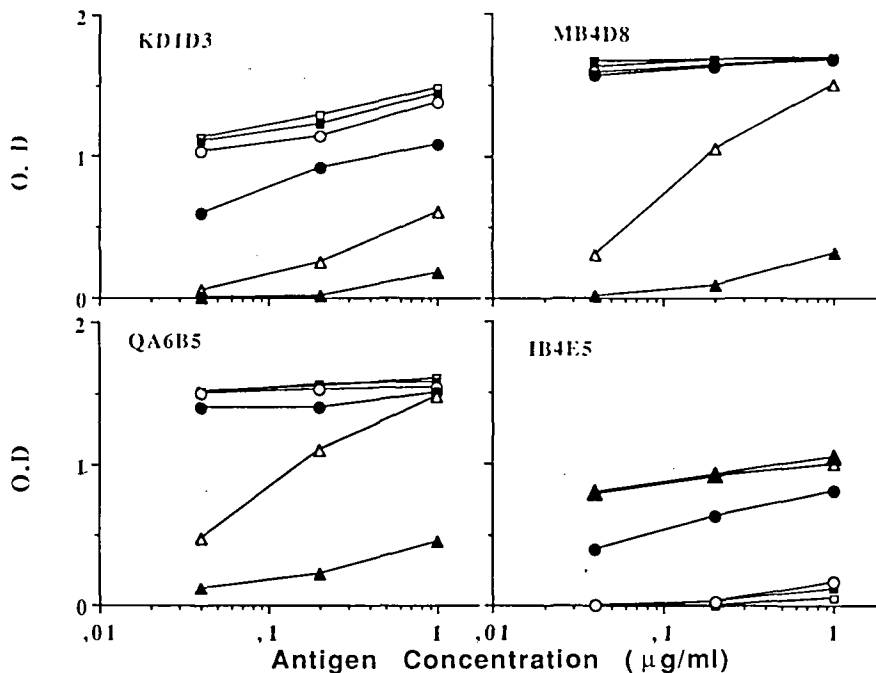


FIG. 5. Effect of different temperature treatments (non-treated, 65, 70, 75, 80 and 85°C) on ovalbumin as studied with four monoclonal antibodies. Binding was evaluated by the sandwich EIA using plates coated with MAb as in Figure 1. (□) N; (■) 65°C; (○) 70°C; (●) 75°C; (△) 80°C; (▲) 85°C.

fluorescence analysis. Moreover, we found that specific MABs can distinguish different heat treatments at 65, 70, 75, 80 and 85°C.

The large plakalbumin peptide (PK), which represents 85–90% of the NOA molecule including the N-terminal region (Thompson *et al.*, 1971) exhibited a drastic change of antigenic reactivity against our panel of MABs as compared with NOA. The MABs binding to the NOA did not react with PK, with the exception of AD6C4 and IB4E5 which were able to bind to both molecules: AD6C4 was much more reactive with NOA than with PK while the reverse was true for IB4E5. In contrast, no difference between NOA and PK binding could be detected with polyclonal antibodies (Yokata *et al.*, 1982).

The thermal treatment at 100°C of NOA in solution led to the creation of two types of molecules: monomeric and heat-denatured ovalbumin (mHDOA) which exhibited the same antigenic determinants as NOA, and polymeric heat-denatured ovalbumin (pHDOA) which was identified as the heat-denatured ovalbumin. Thus, either the monomers (1%) reconstitute the native form of ovalbumin after cooling and only the aggregation of molecules (99%) led to a stable structure or the heating process by itself does not modify the rigid structure of the monomeric molecule which is stabilized by a disulphide bond. We would favour the first hypothesis, namely that aggregation stabilizes the modified conformation of the molecule, allowing structural differences to be distinguished between heat treatments at 70, 75 and 80°C.

It has been reported that during the chemical glycosylation of NOA, monomeric glycosylated OA does not differ from NOA, whereas the polymeric form exhibits different antigenic determinants (Kato *et al.*, 1985). Our observation is in agreement with these conclusions.

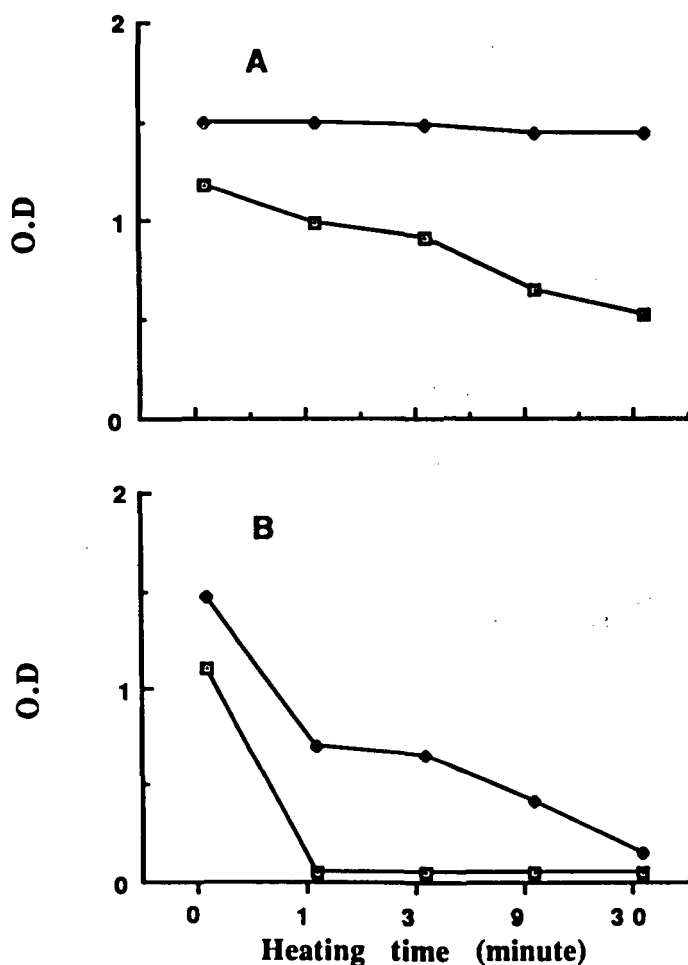


FIG. 6. Effect of the duration of heat treatment on ovalbumin either at 75°C (A) or 80°C (B), measured by QA6B5 MAb. Binding was evaluated by the sandwich EIA using plates coated with QA6B5 MAb, and two concentrations of antigens (3 ng and 100 ng) then with NAc2 Rabbit anti-ovalbumin antibodies and goat anti-rabbit antibodies labelled with peroxidase. (□) 3 ng; (●) 100 ng.

Despite the different physico-chemical properties of PK and pHDOA, such as the molecular weight (Kato & Takagi, 1987), both exhibited almost the same antigenic properties as assessed by MABs. However, a few differences did exist such as the fact that whereas IB4E5 was inhibited to the same extent by PK and pHDOA, G1C6 was much more strongly inhibited by pHDOA than by PK.

It has been reported that the heat denaturation of NOA leads to the formation of polymers with the accompanying exposure of hydrophobic regions (Shimada & Matsushita, 1980). Similarly, two hydrophobic regions are exposed during subtilisin treatment, the first corresponds to the 26–45 residue, which is sequestered during biosynthesis (Meek *et al.*, 1982) and the second corresponds to the 243–253 residue (Lingappa *et al.*, 1979). A consideration of both these and our own observations suggests that the antigenic properties which are common to PK and pHDOA are mediated by epitopes located close to the hydrophobic moiety of these molecules.

Klausner *et al.* (1982) observed a shift in the fluorescence $\lambda_{\max}$ of HDOA monomer from 337 to 341 nm after removal of the denaturant guanidine HCl in which ovalbumin was present. Our results showed that the tryptophan groups in the HDOA after aggregation at room temperature are present in a similar non-polar environment as they were in the NOA molecule. Removal of the peptide fragment from 346 to 385 (which does not contain any tryptophan group) by subtilisin treatment also does not change the non-polar environment around tryptophan groups in PK. The reduced tryptophan fluorescence intensity in both cases probably resulted from the presence of different fluorescence quenching groups in which the tryptophan groups exist.

The aggregation of HDOA molecules is thought to involve the association of intramolecular hydrophobic groups of monomeric HDOA due to their unfavourable free energy of interaction with water. ANS molecules can detect the presence of these hydrophobic areas which are in contact with the aqueous phase, and this is demonstrated by the large shift in the spectrum towards the blue end and the increase in the fluorescence intensity.

Subtilisin treatment of NOA removes the residues 346 to 385 and also removes five acidic and five basic amino acid residues; thus, the net charge of the PK molecule is not altered from that of NOA. Otherwise, an excessive charge could have induced an opening up of the molecule due to electrostatic repulsion, leading to accessibility of some groups to ANS. It has been suggested that removal of the heptapeptide fragment, 346–352, by subtilisin results in a change of protein conformation which is reflected in the formation of plate-like crystals in PK compared with the usual needles obtained for NOA (Smith, 1968). This change in intramolecular interaction probably exposes hydrophobic groups in PK which are detected by ANS.

Recently, Kennet *et al.* (1990) investigated the possibility of evaluating the thermal modification of the structure of lysozyme with MAbs. Testing seven anti-lysozyme MAbs, they found that only one MAb was unable to bind lysozyme which had been heated for 120 min at 95°C. They concluded that this MAb was useful for examining the structural and conformational alteration of protein. Our results showed that MAbs were an effective tool in detecting not only thermal conformational changes but also enzymatic and probably other types of protein denaturations. Moreover, using MAbs we were able to determine the temperature and duration of heat treatment to which a protein had been subjected. It is worthwhile stressing that epitopes remained stable after thermal denaturation, even after cooling the solution.

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