Apparent Discrepancies between Immunochemical Methods used for Ovalbumin Recognition in Food

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ABSTRACT

The specificity of rabbit anti-native ovalbumin antibodies against native ovalbumin (NOA) or heat-denatured ovalbumin (HDOA) was studied by affinity chromatography, ELISA techniques, quantitative immuno-precipitation and HPLC. Affinity-purified antibodies were prepared either by passing antisera successively on an NOA column and an HDOA column, or vice versa. Antisera were found to contain about 85% of antibodies directed against NOA, 7% specific for HDOA and 8% retained by both columns.

It is shown that antibodies specific for NOA fully precipitated NOA in liquid-phase precipitation but were unable to bind NOA in ELISA using NOA-coated plates, while recognising NOA trapped on the solid phase by a first layer of antibodies: ovalbumin was thus completely denatured during interaction with plastic as shown also by the fact that antibodies specific for HDOA reacted with NOA-coated plates.

These results point out the need to modify ELISA tests for monoclonal antibodies selection and for structural studies of food molecules in complex mixtures. However, this observation, which holds true for ovalbumin, might not apply to all antigens.

Key words: Ovalbumin, denaturation, antigenicity, immunoassay, liquid-phase precipitation.

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1 INTRODUCTION

Immunological techniques have been applied to the food industry for the last few years, mainly for the detection of contaminants or additives (Krysinski and Heimsch 1977; Griffiths *et al* 1981, 1984; Hitchcock *et al* 1981; Skerit and Smith 1985; Breton *et al* 1988a). Such methods could have much wider scope in food technology. Unfortunately specific drawbacks can occur in the use of these techniques due to the fact that proteins in foods are mostly denatured and are present in a complex mixture. Denaturation occurs during physical, chemical and enzymic methods of food processing causing changes in the epitopes of protein molecules. Epitopes found on native molecules are often lost and new antigenic determinants specific for the denatured form of a protein are sometimes generated. However, identification of antigen-antibody complexes either by immunoprecipitation or by ELISA is not always easy to interpret: for instance it has recently been found that a protein binding to plastic is somehow denatured, leading to misinterpretation in identifying monoclonal antibodies (Mierendorf and Dimond 1983; Djavadi-Ohanian *et al* 1984; Vaidya *et al* 1985). Some monoclonal antibodies have been found to bind antigen adsorbed to plastic and not to the same antigen in liquid phase and vice versa (Djavadi-Ohanian *et al* 1984). Thus, the application of immunological techniques to the agro-food industries leads to questions such as: should antibodies be raised against the native, denatured or degraded molecule, and is ELISA using antigen-coated plates a suitable test for antigen detection?

In order to monitor food processing, it may be useful to introduce in the raw material test proteins for which antibodies reacting with different epitopes are available; the fate of these different antigenic reactivities in the test protein would then provide information on the likely effects of the processing. In order to answer such questions ovalbumin was used as a model protein in the present work as it is widely used in the food industry. Breton *et al* (1988a) have previously described an indirect ELISA test which allows the detection and quantification of heat-denatured ovalbumin additive in canned mushrooms, using rabbit antibodies raised against native ovalbumin. In the present report, a procedure simplified in order to obtain a qualitative response quickly is described, and a study is presented of antigen-antibody interactions with polyclonal antibodies which allow the native and denatured form of ovalbumin to be detected by ELISA.

2 EXPERIMENTAL

2.1 Heat denaturation of ovalbumin

Hen egg ovalbumin used in this study was from Sigma Chemicals. Heat-denatured ovalbumin (HDOA) was prepared by heating a 1% solution in phosphate-buffered saline (PBS) for 30 min at 100°C. Coagulated ovalbumin was solubilised in 0.1 M NaOH and then neutralised with HCl. HDOA solution was centrifuged at $10\,000 \times g$ for 10 min before use.

For the molecular weight determination, 2 ml of a 5 mg ml^{-1} solution in PBS

was loaded on a Sepharose 4B column (Pharmacia, 2 cm $\times$ 90 cm). The column was calibrated using haemocyanin monomer (Serva, 9×106 amu) and dimer obtained by glutaraldehyde coupling (1.25%, v/v) (Avrameas 1969).

2.2 Immunisation

Three-month-old rabbits were immunised by multiple intradermal injection of antigen–adjuvant mixture (Vaitukaitis *et al* 1971). Antigen was prepared by emulsifying 1 vol saline containing 1 mg native ovalbumin per ml and 1 vol complete Freund's adjuvant. Each rabbit received 1 mg antigen per injection. Two weeks later the procedure was repeated but incomplete adjuvant was used. Animals were bled 5 or 7 days after the last injection. Hyperimmunisation took place once a month by intravenous injection of 1 mg antigen in 0.5 ml of saline, followed by three bleedings on day 5, 10 and 15. Antisera from two rabbits were pooled and used in the subsequent studies.

2.3 Preparation of the immunoadsorbent columns

Ultrogel ACA 22 was activated by the glutaraldehyde technique as indicated by the manufacturer's instructions (IBF Réactifs, France) and then either native ovalbumin (NOA) or heat-denatured ovalbumin (HDOA) was coupled to the activated gel. Ten milligrams of ovalbumin of each form were used per ml of gel. The amount of protein conjugated was estimated photometrically at 280 nm from the difference in concentration before and after reaction with the activated gel. The immunoadsorbent contained about 3 mg of ovalbumin per ml of gel (yield 30%) for both NOA and HDOA.

2.4 Preparation of affinity-purified antibodies

Serum aliquots (2 ml) were loaded on the immunoadsorbent column (1 $\times$ 10 cm) equilibrated in PBS, with flow rate 20 ml h^{-1} . Antibodies were eluted with 0.2 M glycine–HCl buffer pH 2.1 containing 10% v/v dioxane, immediately neutralised with Tris, and then dialysed against PBS in the cold (4°C for 24 h); the column was regenerated by passage of 5 to 10 vol 0.1 M Tris–HCl, pH 8.5, 0.5 M NaCl, and 5 to 10 vol 0.1 M sodium acetate, pH 4.5, 0.5 M NaCl. Purified antibodies were then concentrated to 2–5 mg ml^{-1} and stored at $-20^\circ C$.

When needed, affinity-purified antibodies were conjugated to alkaline phosphatase (P L Biochemicals) using 0.06% glutaraldehyde (Clark and Adams 1977).

2.5 Enzyme immunoassays

Solid-phase assay using antigen adsorbed to a solid support was a modified ELISA from Engvall and Perlmann (1972).

2.5.1 Indirect ELISA

In each well of a microtitre plate (Nunc), 0.2 ml of antigen (200 ng ml^{-1}) of either NOA or HDOA in 0.05 M carbonate buffer, pH 9.6, was added and the plates were incubated for 2 h at 37°C or overnight at 4°C. Coated plates were then washed with PBS containing 0.05% v/v Tween 20. A 1% w/v gelatin solution in carbonate buffer

was added to each well (0.2 ml) for 1 h at 37°C to block remaining protein binding sites. After washings, plates were incubated with 0.2 ml purified rabbit anti-NOA antibodies (200 ng ml⁻¹ in PBS-Tween) and allowed to react for 2 h at 37°C. The plates were then washed and 0.2 ml of goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Laboratories, 1:3000 in PBS-Tween) was added to each well. After a 2-h incubation at 37°C, 0.2 ml of freshly prepared substrate solution containing 50 µl ABTS (Boehringer Mannheim, 2,2'-azino-di(3-ethylbenzthiazoline sulphonate-6)) stock solution (100 mg 4.5 ml⁻¹ distilled water) and 40 µl H₂O₂ in 10 ml 0.05 M citrate buffer pH 4 was added to each well. The reaction was stopped by addition of 20 µl 10% SDS and the absorbance was measured at 405 nm using a Bio-Tek EL 307 plate reader. For each sample, controls for non-specific binding of anti-NOA antibodies and enzyme-labelled antibodies were incorporated.

2.5.2 Competitive indirect ELISA

Purified anti-NOA antibodies (200 ng ml⁻¹ in PBS-Tween) were incubated in test tubes with increasing concentrations of either NOA or HDOA for 2 h at 37°C. Aliquots of 0.2 ml of this mixture were then added in the microplate wells coated with 0.2 ml of either NOA or HDOA (200 ng ml⁻¹) and allowed to react overnight at 4°C. After removing unbound reagents by washings, antigen-antibody reaction was revealed by addition of IgG-peroxidase conjugate, and colour-forming product was measured as described earlier.

2.5.3 Double antibody sandwich ELISA

Microtitre plates were coated overnight at 4°C with 0.2 ml of rabbit anti-NOA antiserum (1:100 in carbonate buffer). Unbound antibodies were removed by washing and gelatin was added as described above. After washings, plates were incubated at 37°C with 0.2 ml of either NOA or HDOA in a concentration range from 1 ng to 1 µg ml⁻¹. Unbound antigen was washed away and affinity-purified anti-NOA antibodies (10 µg ml⁻¹) coupled to alkaline phosphatase were added for 2 h at 37°C. After further washings, the bound enzyme conjugate was detected by adding 0.2 ml of *p*-nitrophenylphosphate (Sigma) at 1 mg ml⁻¹ in 0.1 M diethanolamine buffer, pH 9.8. The reaction was stopped by addition of 50 µl 3 M NaOH and the absorbances were read at 405 nm.

2.5.4 Direct ELISA using antigen-coated plates

The same procedure as double antibody sandwich ELISA was used except that NOA and HDOA were adsorbed to plastic and then plates were incubated with affinity purified anti-NOA antibodies coupled to alkaline phosphatase.

2.6 Quantitative immunoprecipitation

Standard procedures were employed (Kabat 1961). Purified anti-NOA antibodies (300 µg) were mixed with increasing concentrations of either NOA or HDOA, in a final volume of 1 ml. Samples were first incubated for 1 h at room temperature, and then for 3 days at 4°C. Resultant precipitates were washed with cold PBS and centrifuged at 10 000 × *g* for 5 min, repeating the cycle three times, and then dissolved in 0.3 ml of 0.1 M NaOH. The amount of precipitated proteins was

determined by the method of Lowry *et al* (1951). A correction for non-specific precipitation was made by analysing controls containing only antibody or antigen. Supernates were analysed by HPLC and their residual antibody activity by indirect ELISA with either NOA or HDOA bound to solid phase.

2.7 High performance liquid chromatography (HPLC)

Analyses were carried out with a Spectra Physics model 8700 liquid chromatograph equipped with a UV/visible SP 8440 variable wavelength detector connected to a 4200 computing integrator. The gel filtration HPLC column TSK G 3000 SW (600 × 7.5 mm) with molecular weight range separation for protein: 10^3 – 3×10^5 was used. The eluant used was 0.175 M NaCl at pH adjusted to 7. The flow rate was 1 ml min⁻¹. The eluates were monitored at 220 nm. A 100-μl sample was injected in the column.

2.8 Qualitative determination of OA additive in canned mushrooms by indirect ELISA

Experimental canned mushrooms were treated with ovalbumin at concentrations ranging from 1 to 10%, and then were processed as usual. For comparison cans obtained from different supermarkets were tested.

Ten grams of drained mushrooms in 20 ml cold PBS were homogenised three times for 20 s in a blender (Sorvall omni-mixer) at 10 000 rpm. The homogenate was collected, diluted twice in PBS and then centrifuged for 20 min at $4000 \times g$ to obtain a clear supernate. Indirect ELISA tests using antigen-coated plates were performed as previously described (Breton *et al* 1988a).

2.9 Protein analysis

Protein concentrations were determined by ultraviolet spectrophotometry at 280 nm, or by the method of Lowry *et al* (1951), using a bovine serum albumin solution as standard.

3 RESULTS

3.1 Characterisation of native and heat-denatured ovalbumin

Ovalbumin used in these studies was obtained from Sigma and gave only one band on SDS–polyacrylamide gel electrophoresis (Breton *et al* 1988b). By HPLC analysis on a TSK G 3000 SW column, NOA exhibited a single peak corresponding to a molecular weight of 45 000 as expected (data not shown). Coagulated ovalbumin solubilised in NaOH 0.1 M (HDOA) was in an aggregated form which did not enter the separation gel in SDS–PAGE (12% w/v acrylamide concentration) unless previously treated by 2-mercaptoethanol (Breton *et al* 1988b). This was confirmed by elution of HDOA in the void volume of the TSK G 3000 SW column (see below).

By gel filtration on Sepharose 4B with molecular weight range separation for protein up to 20×10^6 , the molecular weight of HDOA was estimated as about 18×10^6 (Fig 1).

Fig 1. Determination of HDOA molecular weight by gel filtration using a Sepharose 4B column, as described in Section 2.

3.2 Heterogeneity of affinity-purified antibodies

An aliquot of 2 ml rabbit anti-NOA serum was loaded on an NOA column or on an HDOA column. Antibodies that were not retained were loaded on either an HDOA or an NOA column as shown in Fig 2. Antibody recovery was determined from absorbance measurements at 280 nm of proteins eluted with pH 2.1 buffer.

It can be seen from Fig 2 that, using hyperimmune sera, the NOA column retained 1.56 mg Ac1 antibodies, whereas 0.13 mg Ac4 antibodies were still present in the flow-through antibodies which were totally retained on an HDOA column. Vice versa, whereas only 0.28 mg Ac2 antibodies were retained on a HDOA column, 1.45 mg Ac3 antibodies were recovered from the NOA column. No antibody activity was found by indirect ELISA in the second flow-through fluids. The sum of Ac1 + Ac4 antibodies was equivalent to Ac2 + Ac3 antibodies. From these results we can estimate that about 85% of antibodies were directed against NOA, 7% against HDOA and 8% recognised both forms of the molecule.

A control with sera from preimmunised rabbits was done to ascertain that no unrelated proteins were retained on the immunoadsorbents.

3.3 Analysis of affinity-purified antibodies by competitive indirect ELISA test

As shown in Table 1, in the absence of competitor the four antibody specificities described earlier recognised differently either NOA or HDOA. Antibodies retained on the HDOA column (Ac2, Ac4) gave a higher signal than those retained on the NOA column (Ac1, Ac3) either with NOA or HDOA used as coating antigens; the lowest absorbance was found with Ac3.

By competition it was found that HDOA was much more potent than NOA at inhibiting the 50% absorbance signal given by indirect ELISA test; the amount of

Fig 2. Analysis of anti-native ovalbumin antibodies by affinity chromatography. Rabbit antiserum was loaded on native OA column (NOA) or on heat denatured OA column (HDOA) as indicated on the figure. Bound antibodies were desorbed as described in Section 2.

TABLE 1
Competitive indirect ELISA of affinity-purified antibodies with native or heat-denatured ovalbumin

Antibody	NOA adsorbed on solid phase			HDOA adsorbed on solid phase		
	No competitor	Competition with:		No competitor	Competition with:	
		NOA	HDOA		NOA	HDOA
Ac1	0.8 ^a	0.04 ^b	0.006 ^b	0.98 ^a	4 ^b	0.02 ^b
Ac2	1.4	4	0.13	1.46	No inhibition	0.3
Ac3	0.23	0.3	0.01	0.5	1.5	0.02
Ac4	1.06	1.25	0.009	1.26	4	0.025

^a Absorbance values measured after 15 min incubation of peroxidase substrate.

^b Results are expressed in inhibitor concentration ($\mu\text{g ml}^{-1}$) giving 50% inhibition in ELISA. In all experiments each antibody was analysed in triplicate.

NOA required is 10- to 200-fold higher than the amount of HDOA exhibiting the same inhibition. These results suggested that NOA bound on polystyrene support behaved as HDOA did. It was thus questionable whether the epitopes corresponding to the native state were preserved when antigens were adsorbed to a solid phase. To answer this question Ac1 antibodies (retained on the NOA column) were analysed by quantitative immunoprecipitation in liquid phase.

3.4 Quantitative immunoprecipitation in liquid phase

Ac1 antibodies (300 μg) were incubated with increasing concentrations of either NOA or HDOA for 3 days at 4°C. With NOA the classical curve with a maximum precipitation of antigen-antibody complexes for 40 μg of added antigen was observed (Fig 3A). In contrast HDOA, when mixed with Ac1 antibodies, exhibited a slow increase of antigen-antibody precipitates up to 120 μg of added antigen followed by a slow decrease of precipitates when more antigen was added (Fig 3A). At first sight this would mean that while all antibody specificities in Ac1 were

Fig 3. Analysis of Ac1 antibody fraction by quantitative immunoprecipitation in liquid phase. 300 μg antibodies were mixed with increasing concentrations of either NOA (▲) or HDOA (●) as described in Section 2. The amount of precipitated proteins was determined by the method of Lowry *et al* (1951), using bovine serum albumin as standard (A). Free antibodies in supernate were measured by indirect ELISA using either NOA-coated plates (B) or HDOA-coated plates (C).

recognised by NOA allowing a complete precipitation, only a small amount of these specificities cross-reacted with HDOA leading to an incomplete precipitation.

The presence of residual antibody activity in supernates was measured by indirect ELISA with either NOA (Fig 3B) or HDOA (Fig 3C) adsorbed on solid phase. On Fig 3B it can be seen that, after precipitation with NOA, residual antibody activity dropped almost to zero at equivalence and then increased up to 30% for 150 μg of added antigen. After precipitation with HDOA, 15% antibody activity was observed whatever the antigen concentration used.

On Fig 3C it can be seen that, after precipitation with NOA, residual antibody activity at equivalence dropped almost to 15% and then increased up to 40%. No antibody activity was detected in supernates from precipitates with HDOA whatever the antigen concentration up to 100 $\mu\text{g ml}^{-1}$, whereas, in antigen excess, 15% activity was still detectable.

Thus low amounts of denatured antigen (ie less than 20 $\mu\text{g ml}^{-1}$) were sufficient to precipitate all of the antibodies reacting in the indirect ELISA test, whereas 40 μg of NOA was required to observe the same result. This was in accordance with the observation that HDOA was apparently a better competitor in indirect ELISA.

However, during immunoprecipitation in the presence of HDOA, either specific antibodies against NOA were unable to bind the denatured antigen and stayed as free antibodies or they should have bound HDOA leading to soluble complexes. In order to decide which hypothesis was correct, HPLC was used to analyse supernates from immunoprecipitation tests in liquid phase.

By using a gel filtration TSK G 3000 SW column, it can be seen (Fig 4A) that in

Fig 4. HPLC analysis of supernate from the immunoprecipitation test in liquid phase. Supernate at equivalence from the immunoprecipitation tests with NOA (A) and with HDOA (B), and supernates in antigen excess zone with NOA (C) and HDOA (D), were analysed using a TSK G 3000 SW column.

Elution times of NOA, HDOA and antibodies were 17.5, 10.2 and 16.4 min, respectively.

the presence of NOA at equivalence a detectable peak of neither antigen nor antibody was found; in antigen excess (Fig 4C) two peaks were detected corresponding to antigen-antibody complexes (12.4 min) and to free antigen (17.6 min), respectively.

In contrast to HDOA at equivalence (Fig 4B), a large peak of free antibody (16.5 min) representing about 90% of added antibodies was found with a very small peak of either denatured antigen or antigen-antibody complexes (10.2 min) which was excluded from the column; in antigen excess (Fig 4D) a large peak of antigen (10.1 min) was seen with again the same large peak of free antibody (16.4 min).

Thus almost all antibodies in Ac1 could bind and precipitate NOA in solution whereas only a small proportion of them could bind and precipitate HDOA. Furthermore, NOA bound on solid phase was denatured since free antibodies in the immunoprecipitation test with HDOA failed to recognise NOA in indirect ELISA whereas they recognised NOA in solution.

Fig 5. Comparison of direct (—) and double antibody sandwich (---) ELISA for the detection of NOA (●) and HDOA (▲) with Ac1 (A), Ac2 (B) and Ac3 (C) antibodies coupled to alkaline phosphatase. In direct ELISA tests, NOA and HDOA were adsorbed directly to the solid phase. In the double antibody sandwich ELISA test, NOA and HDOA were bound to rabbit anti-NOA antibodies adsorbed to the solid phase (see Section 2).

In order to identify specific antibodies against NOA, Ac1, Ac2 and Ac3 were coupled to alkaline phosphatase and used in direct and double antibody sandwich ELISA tests. It can be seen on Fig 5A that both NOA and HDOA were recognised by Ac1 antibodies in both such tests. Ac2 antibodies recognised HDOA as well by both techniques (Fig 5B), but not NOA in the range of concentrations used. Ac3 antibodies which must be strictly specific for NOA did not recognise NOA in the direct ELISA, but recognised fully bound NOA in the double antibody sandwich ELISA; no binding was observed with HDOA in both tests (Fig 5C).

Finally, the amounts of antibodies specific against NOA in Ac1 and Ac3 (Figs 5A and 5C, respectively) are similar as shown by superimposable curves which confirm the previous conclusion that about 90% of Ac1 antibodies were directed against NOA while remaining antibodies recognised preferentially a denatured form of ovalbumin (HDOA or NOA denatured by interaction with plastic).

3.5 Detection of ovalbumin additive in canned mushrooms

As shown in Table 2, a single protein extraction in PBS is sufficient to detect OA additive in canned mushrooms. Thus a qualitative response can be obtained quickly inasmuch as incubation times in each step of the ELISA test can be shortened to produce the result within a day. From these results it can be concluded that F3 and F6 contain at least 5% OA, whereas F5 and F41 contain 1% or less of OA. However, the analysis of juices sometimes gave positive results but in many cases the values were too low to be significant. These variations may be attributed to differences in industrial processes due to 'blanching'.

TABLE 2
Detection of ovalbumin in canned mushrooms by indirect ELISA

	Absorbance, 405 nm	
	PBS extracts	Juices
Experimental cans ^a		
Control	0.05	0
1% OA	1.26	0.04
5% OA	1.40	0.11
10% OA	1.44	0.15
Commercial cans ^b		
F2	0.51	0.02
F3	1.34	0.06
F6	1.17	0.01
F6	1.52	0.07
F41	1.05	0.54

Microplates were coated with PBS extracts or juices (undiluted) and ovalbumin was revealed by addition of $0.5 \mu\text{g ml}^{-1}$ of Ac1 antibodies. Results are the mean of at least three determinations.

^a Canned mushrooms treated with known amounts of ovalbumin.

^b Positive commercial cans taken from different supermarkets.

4 DISCUSSION

In a previous paper (Breton *et al* 1988b), it was shown, by indirect ELISA and competitive indirect ELISA, that HDOA was detected at lower concentration than NOA, with rabbit anti-NOA polyclonal antibodies. Moreover, by quantitative immunoprecipitation it was suggested that soluble HDOA-antibody complexes might explain the disappearance of antibody activity in the supernate revealed by ELISA.

In this report, binding of antibodies raised in rabbits against native ovalbumin has been studied with either NOA or HDOA using different immunochemical procedures. It was shown that ovalbumin after heat coagulation and resolubilisation exhibited a very high molecular weight close to 18×10^6 instead of 45×10^3 under its native form, as judged by chromatographic analysis on Sepharose 4B.

By affinity chromatography on either NOA or HDOA column, different specificities were found among polyclonal antibodies: 85% of those present in rabbit antisera were strictly specific for NOA while 7% were strictly specific for HDOA and 8% were retained by both columns. It might be either that a small fraction of NOA used as antigen could be denatured and cross-reacted with HDOA or that during the mixing with Freund's adjuvant some of the OA molecules are denatured: the authors favour the first hypothesis as native ovalbumin often contains a small fraction of denatured molecules (Kilshaw *et al* 1986). A third hypothesis would be that a common epitope was revealed by the undenatured glycosylated moiety present on NOA and HDOA. It remains to be proven that ovalbumin denaturation leads to similar epitopes whatever the means of denaturation used: noteworthy is the fact that Ac2 antibodies recognise only NOA adsorbed on plastic and bind to HDOA either in solution or adsorbed on plastic.

One of the most striking results was that antibodies specific for NOA (Ac3) exhibited an extremely weak binding on NOA in indirect ELISA test as compared with antibody fractions specific for HDOA (Ac4) or containing mostly antibodies specific for HDOA (Ac2). This observation was confirmed by competitive indirect ELISA showing, for all four types of antibodies, that HDOA was a better competitor than NOA, thus suggesting that NOA on plastic was completely denatured. Moreover, by labelling either Ac1, Ac2 or Ac3 antibodies with alkaline phosphatase, it was shown that only Ac1 and Ac2 antibodies were able to bind HDOA in direct and double antibody sandwich ELISA tests. The higher binding observed for HDOA compared with NOA could be explained by assuming that antibodies were bound on HDOA by their two binding sites, due to repetition of epitopes on to the 18×10^6 amu polymer, whereas they were bound by only one binding site on NOA: this would be a plausible explanation for the slopes being found to be steeper for both Ac1 and Ac2 for HDOA than for NOA (Figs 5A and 5B). It was not possible to harvest a sufficient yield of Ac4 to make the same study.

ELISA has been and is still widely used as a screening test to detect monoclonal antibodies, but some drawbacks in the use of this technique need to be emphasised. For instance, some monoclonal antibodies against the B2 subunit of *Escherichia coli* tryptophan synthase reacted slowly with the enzyme in solution whereas they

recognised the antigen quite rapidly when adsorbed on to the ELISA plate (Djavadi-Ohanian *et al* 1984), and antibodies which bound slowly to the native B2 subunit in solution reacted rapidly with denatured antigen (Friguet *et al* 1984). Similarly, by comparing seven different ELISA procedures to study the structure of tobacco mosaic virus, it was found that, depending upon the test used, different monoclonal antibodies with different specificities could be identified (Al Moudallal *et al* 1984). Using a modification of the ELISA procedure (Kemeny *et al* 1985; Smith and Wilson 1986), it was shown that antigen denaturation could be avoided allowing a selective detection of monoclonal antibodies reacting with the native antigen. In a recent paper (Kilshaw *et al* 1986), it was shown that ovalbumin behaved as the B2 subunit from *E coli* tryptophan synthase, developing a relatively high degree of denaturation by interaction on solid phase: this observation was supported by the study of four hybridoma lines synthesising specific antibodies.

The present study of the interaction of Ac1 antibodies with either NOA or HDOA emphasises the fact that only a very small proportion among them interacted with HDOA by indirect and competitive indirect ELISA: however, in order to detect a perfectly native molecule, it was necessary to make this study in solution, using the classical quantitative immunoprecipitation method (Kabat 1961). It was observed that almost all Ac1 antibodies were precipitated at equivalence with 40 μ g of NOA as shown by HPLC analysis of supernates. In contrast with HDOA, a large peak of free antibodies, representing about 90% of Ac1 antibodies, was present in the supernate as much at equivalence as in antigen excess. This observation fits with our previous data that in Ac1 there is about 8% of common antibodies for NOA and HDOA. These antibodies which present a high affinity for HDOA might be the only ones that are revealed in indirect ELISA tests with NOA used as coating antigen. The results explain why Ac1 is able to reveal HDOA in mushroom extracts as shown previously (Breton *et al* 1988b).

Thus it can be concluded that indirect ELISA and competitive indirect ELISA are ineffective as screening methods to detect monoclonal antibodies directed against NOA as about 90% among them will not be identified. Here, by comparing quantitative immunoprecipitation and ELISA tests, it was possible to quantify by HPLC the proportion of antibodies in rabbit antisera unable to react in ELISA while binding the antigen in solution. It was shown that by either binding the antigen through a monoclonal antibody adsorbed to ELISA plates (Kilshaw *et al* 1986), or polyclonal antibodies (Al Moudallal *et al* 1984; Kemeny *et al* 1985; Smith and Wilson 1986), or the use of glutaraldehyde (Holt *et al* 1984; Sedgwick and Holt 1986), the drawbacks of ELISA with antigen-coated plates could be avoided. Work is now in progress in order to identify, with monoclonal antibodies, specific epitopes present on the native protein but disappearing during thermodenaturation or other denaturation processes.

It might be of interest for the agro-food industries to detect epitopes specific for different steps of food processing, so as to correlate the structural changes that occur during industrial processes (eg fermentation, cooking, extrusion etc) and the technological quality of finished products. Only the use of specific reagents such as monoclonal antibodies could lead to such an achievement in complex media. Work is now in progress to study such structural modifications in ovalbumin using monoclonal antibodies.

Besides the study of identification of the different epitopes on NOA and HDOA, the authors wished to test the possible use of polyclonal antibodies for the detection of HDOA in foods. It was shown, using experimental canned mushrooms with added OA, that several commercial canned mushrooms indeed contained OA in the range 0.5–5 %. The low levels of OA found in juices is due to 'blanching', consisting of pre-heating at 90°C and washing before the cans were filled up with a citrate solution. However, the high levels of OA found in mushroom extracts give a fair picture of the amount of added OA. It is shown here that a simplified procedure of extraction is reliable in comparison with that previously described (Breton *et al* 1988a).

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