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Analytica Chimica Acta 405 (2000) 255–265

ANALYTICA CHIMICA ACTA

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Sensitive detection of triazine and phenylurea pesticides in pure organic solvent by enzyme linked immunosorbent assay (ELISA): stabilities, solubilities and sensitivities

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Received 20 May 1999; received in revised form 31 August 1999; accepted 5 September 1999

Abstract

Triazine and phenylurea pesticides could be detected in pure ethanol and hexane by enzyme linked immunosorbent assay (ELISA), using microtiter plates for covalent immobilization of the respective monoclonal antibodies K4E7 and B76-BF5. Polystyrene microtiter plates were found to be resistant against several organic solvents. From those, hexane and ethanol were chosen, as they are common extraction solvents for many pesticides. For ethanol, dependence of solvent concentration on antibody and marker enzyme stability and antibody affinity was investigated. The thermal stability had a minimum at 50–80% ethanol. At low temperatures, stabilities were sufficiently high for ELISA experiments in the whole ethanol concentration range from 0 to 99% v/v. The test midpoints IC_{50} of the immunoassays were at least 30-fold higher, when the antigen was presented in ethanol, compared with pure water. However, a lower test midpoint was observed when the phenylurea antigen was dissolved in hexane instead of water. Detection limits below 0.2 ng ml^{-1} were obtained for both atrazine and phenylurea OC-2 in hexane. The sensitivities and cross-reactivities in the three solvents water, ethanol and hexane could be correlated with the solubilities of the antigens. For example, the phenylurea antigens were less soluble in hexane than in water, and therefore, their binding to the antibody was improved in hexane. These results can be explained by the hydrophobic effect. The method is potentially applicable to hydrophobic pesticides, which have to be extracted with a hydrophobic solvent, e.g. hexane.

Keywords: Organic solvent; Hexane; Ethanol; ELISA; Monoclonal antibody; Stability; Affinity; Atrazine; Phenylurea; Pesticides; Solubility

1. Introduction

The preparation of environmental samples for pesticide analysis often includes an extraction step with organic solvents. Such solvents are compatible with instrumental analysis, e.g. liquid chromatography

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0003-2670/00/$ – see front matter
PII: S0003-2670(99)00685-6

(LC), but in immunoassays, they are either avoided or diluted to low concentrations. There are only few publications describing immunoassays in water-miscible solvents up to 50%, in connection with matrix effects [1,2], effects on enzyme label [3], antibody affinity [4] or binding kinetics [5].

The influence of water miscible solvents on proteins has been studied in two directions: (a) starting with water and increasing the solvent concentration; (b) starting with pure solvent and increasing the water concentration. The first approach revealed that proteins generally loose activity (enzymes) or affinity (antibodies) and stability with increasing ethanol concentration [6,7], although some increase in affinity and stability was described for solvents in the concentration range 5–25% v/v [8–10]. Also, examples of increased enzyme activity are described for such solvent concentrations [11]. The second approach was chosen to determine the minimum amount of water (or water activity a_w) necessary for the activity of an enzyme in an organic solvent [12,13]. Enzymes in low water systems have been reviewed [14]. In a recent review, the activity and stability of enzymes in 0% to nearly 100% solvents in water was discussed [11]. Here, we describe the effects of ethanol in the range 0–97% v/v on both the antibody stability and the affinity, in order to find out under which conditions extracted analytes are best analyzed by immunoassays.

Antibodies have been also shown to bind their analyte in water immiscible organic solvents [15,16], but very few proposals have been made to date for the detection of analytes in organic solvents like hexane [17,18]. Here, we show that common enzyme linked immunosorbent assay (ELISA) procedures can be used with a few modifications for the highly sensitive detection of herbicides in hexane.

2. Experimental

2.1. Chemicals

Production and characterization of monoclonal antibody B76-BF5 and synthesis of phenylurea derivatives OC-1 and OC-2 and derivatives thereof have been described elsewhere [19]. Affinities and binding kinetics of this antibody have been described previously [20]. OC-1 is *N*-(2-aminobenzyl)-*N*′-4-chlorophenyl-urea and OC-2 is *N*-(2-*N*-chloroacetyl-aminobenzyl)-*N*′-4-chlorophenyl-urea. 2 mg ml^{-1} stock solutions in dimethyl sulfoxide (DMSO) were prepared and diluted to 1 mM in ethanol. Monoclonal antibody K4E7 against atrazine was obtained from Th. Giersch and B. Hock, Technical Univ. München, Germany. 4-Chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-aminocaproic acid) was a gift and triazine standards were purchased from Riedel deHaen (Seelze, Germany). Triazine stock solutions of 1 mg ml^{-1} were prepared in ethanol. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), *N*-hydroxy-succinimide (NHS), 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB), 2,2′-azino-di[3-ethyl-benzthiazoline-sulfonate(6)] (ABTS), 2-iminothiolane and Tween 20 were from Sigma (Deisenhofen, Germany), and hydrogen peroxide from Chemapol (Praha, Czech Republic). HBS (10 mM Hepes with 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20 at pH 7.4) was the running buffer for Biacore experiments; PBS (20 mM potassium phosphate, 150 mM NaCl pH 7.2) was the standard buffer for ELISA. PBS-T was PBS supplemented with 0.05% v/v Tween 20, PBS-TB contained in addition 0.5% w/v bovine serum albumin. Ethanol absolute, dimethylformamide (DMF), tetrahydrofuran (THF) and acetonitrile were from Merck (Darmstadt, Germany), and *n*-hexane HPLC grade from Roth (Karlsruhe, Germany). 2-(5-Norbornene-2, 3-dicarboximido)-1,1,3,3-tetramethyluronium tetra-fluoroborate (TNTU) was purchased from Calbiochem-Novabiochem, Bad Soden, Germany. Microtiter plates MaxiSorp and CovaLink (Nunc, Roskilde, Denmark) were used. Horseradish peroxidase (HRP) EIA grade was obtained from Boehringer Mannheim (Germany).

2.2. Determination of antibody stability with Biacore

Antibody (100 nM) was preincubated in water–ethanol mixtures at various temperatures and incubation times. The phosphate buffer concentration was less than 0.1 mM due to the buffer content of lyophilized antibody. Residual binding ability of preincubated antibody was determined with the Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). The preincubated mixture was diluted 10-fold

in HBS buffer and 50 μl of this dilution injected into a Biacore flowcell containing immobilized atrazine. Residual binding ability was expressed as the apparent concentration of non-denatured antibody, which was calculated by comparison of the initial slope of signal increase with a standard curve for increasing concentrations of antibody. Therefore, the initial binding of antibody to the chip surface containing a high ligand density is measured. The association rate is mass transport limited if the association rate constant k_a is higher than $10^{-5}\,M^{-1}\,s^{-1}$ [21], but more or less kinetically controlled if k_a is low. Therefore, we cannot distinguish if the number of antibodies retaining binding ability is reduced or if the affinity of the binding sites is reduced.

Atrazine was immobilized onto the flowcell of a CM5-chip as follows. The flowcell was first activated with EDC/NHS and aminated with ethylenediamine according to the BIA-Application handbook. 4-Chloro-6-(isopropylamino)-1,3,5-triazine-2-(6-amino-caproic acid) was activated with TNTU in DMF/THF (5 mg atrazine-derivative in 0.5 ml THF, 6.1 mg TNTU in 0.1 ml DMF and 2.5 μl triethylamine for pH adjustment). After 20 h incubation at 20°C, the mixture was dropped onto the chip containing the aminated surface and incubated for 20 min. Ethanolamine (1 M, pH 8) was used to block remaining activated sites on the chip. The short immobilization time of 20 min was used, as the plastic support was slowly dissolved by the solvents.

2.3. Determination of antigen solubility

Solubility was determined by dissolving excess antigen in 10 ml solvent and mixing with a magnetic stirrer for 24 h at 20°C. The suspension was centrifuged and the supernatant measured photometrically. Ethanol samples had to be diluted with water, and hexane samples of triazines were diluted with ethanol. Appropriately diluted standards of antigen in the solvents were used as controls. Four to six independent stock solutions of triazines were prepared for the determination or the molar extinction coefficients ε ($mM^{-1}\,cm^{-1}$). The following ε values were determined: 48.7 ± 7.9 (atrazine in water, 222 nm, $n = 4$), 43.9 ± 6.5 (propazine in water, 222 nm, $n = 6$), 24.2 (OC-1 in water, 243 nm), 21.8 (OC-2 in water, 245 nm), 36.2 (OC-1 in hexane, 246 nm), 41.9 (OC-2 in hexane, 246 nm). The molar extinction coefficients of the triazines were not significantly effected by ethanol and hexane.

2.4. Synthesis of conjugates

Atrazine–peroxidase conjugate was prepared as described previously [22]. OC-2–peroxidase conjugate was synthesized by the following procedure: 1 mg of HRP in 0.1 M $NaHCO_3$ was modified with 1 mg iminothiolane and incubated for 20 min at room temperature. The thiolated enzyme was passed over Sephadex G25 (NAP-5 column, Pharmacia, Uppsala, Sweden). 0.25 mg OC-2 in 5 μl DMSO was added and incubated for 4 h. Excess reagents were removed by passage over NAP-5.

2.5. ELISA procedures

2.5.1. Sequential saturation assay

The sequential saturation immunoassay was based on the coating antibody format which requires sequential incubation of antigen and antigen–enzyme conjugate. All incubation steps were performed at room temperature, unless indicated. 100 μl antibody (0.45 μg = 3 pmol) per well were used for immobilization onto CovaLink microtiter plates in the presence of EDC (314 nmol) and NHS (87 nmol) for 2 h. Ethanolamine (0.5 M pH 8.0, 150 μl) was used for blocking for another hour. After washing three times with PBS-T and once with aqua dest, residual water was removed with 10 μl pipettes, followed by air drying for several minutes. 150 μl antigen dilutions in the respective solvent (from 1 mM stock solution in ethanol) were incubated for 1 h. The plates were covered with a sealing foil (Nunc, Roskilde, Denmark) to avoid evaporation of the solvents. After washing three times with PBS-T, 100 μl antigen–peroxidase conjugate (1E-4 diluted) in PBS-TB was incubated for 15 min. Plates were washed again three times with PBS-T and 100 μl substrate solution (1 mM TMB, 1 mM H_2O_2 in 10 mM acetate pH 4.0) added. The colour development was recorded by a microtiter plate reader (Multiskan RC, Labsystems, Finland) at 380 nm. Mean values were obtained from four wells per antigen concentration. Semilogarithmic plots were

prepared from the resultant data. The curves were fitted by a four-parameter log-logistic model (Microcal Origin, sigmoidal fit). Error bars indicate standard deviation.

2.5.2. Competitive assay

Haptens and the corresponding conjugates were incubated together for 1 h in buffer or ethanol, in contrast to the sequential saturation assay. All other steps were as described above.

2.6. Peroxidase assay

HRP was assayed in the enzyme stability experiments using a substrate mixture consisting of citrate buffer pH 4.0 (10 mM), ABTS and hydrogen peroxide (both 1 mM). The increase in absorbance was recorded at 415 nm.

3. Results and discussion

3.1. Solvent screening

3.1.1. Stability of microtiter plate against organic solvents

Fifteen commonly used organic solvents were tested with respect to their ability to solve polystyrene microtiter plates (MaxiSorp). Due to their high volatility, acetonitrile, THF, acetone and pentane were not taken into account. The plates were dissolved by toluene, dichloromethane, octanol, cyclohexane, DMF, THF stable against acetonitrile, DMSO, methanol, ethanol, isopropanol, butanol, hexane, heptane, octane.

3.1.2. Relative affinity and stability of antibodies

Antibody coated CovaLink plates were treated with pure solvent or atrazine (1 ng ml^{-1}) in solvent for 1 h. Controls were used without antibody. The binding of antibodies in the various solvents is shown in Table 1.

After treatment with all solvents, with the exception of DMSO, enough non-denatured antibody remained, so that inhibition assays could be performed. It was also found (as in later experiments) that Tween 20 has a stabilizing effect. A lower degree of competition (signal reduction) in water, compared with buffer, was also observed for the phenylurea antibody, but not studied in detail. The only solvent showing competition at that low antigen concentration was hexane.

3.2. Ethanol effects on antibody and marker enzyme stability

Stability was defined in our experiments as residual enzyme activity or the residual binding of antibodies to an excess of immobilized antigen (Biacore). Therefore, in the Biacore experiments, reduced association is the measure for stability. Conformation changes or aggregation were not measured in these experiments. Apparent changes in activity caused by adsorption processes will be discussed.

3.2.1. Stability of antibodies in ethanol–water mixtures

The stability of Mab K4E7 was determined using Biacore. Typical stability profiles for Mab K4E7 at 20 and 30°C are shown in Fig. 1. Ethanol up to 20% apparently stabilizes the antibody. A stability minimum is observed between 40 and 80% ethanol. Above 80% ethanol, the stability of the antibody increases again.

After 1 h of incubation of antibody in HBS, water, 50 and 99% ethanol at 20°C, the residual binding ability was 99, 75, 72 and 73%, respectively.

The higher stability in HBS, as compared with water (containing less than 0.1 mM buffer) may be due to the difference in ionic strength and due to the presence of Tween 20 (0.005%), which is known to stabilize proteins (see also above, Section 3.1.2 and Table 1). Indeed, stabilities in PBS buffer without Tween were nearly the same as in water (data not shown). Possibly, the proteins tend to aggregate or adsorb to plastic surfaces in water, and 20% ethanol or low detergent concentration prevent this aggregation or adsorption. It turned out that there was an initial decrease in binding ability within the first hours of antibody incubation, not only in water but also in high ethanol concentrations, while the binding ability remained unchanged after prolonged incubation (Fig. 2). Probably, this loss of binding is due to irreversible adsorption of antibody to the tube wall or due to aggregation processes. It can, therefore, be concluded that the observed 'denaturation' is a multi-step process.

The different principle effects of polar solvents on proteins have been described [11]. Generally, the en-

Table 1
Relative stability and affinity of Mab K4E7 in various solvents at 22°C. Values are corrected by controls without antibody. Standard error was less than 5% ($n = 3$)

Medium	log P[a]	Response (1 ng ml^{-1} atrazine)/response (without atrazine)	Response (solvent)/response (PBS-T)
		Measure of affinity	Measure of stability
PBS-T	−2.7[b]	0.34	1
PBS	−2.7[b]	0.41	0.54
H_2O	−2.7	0.78	0.55
DMSO	−1.3	–[c]	0.016
Ethanol	−0.23	1.02	0.53
Isopropanol	0.28	1.17	0.44
Butanol	0.8	1.04	0.33
Hexane	3.5	0.84	0.32

[a] log P is the logarithm of the coefficient of partition between water and octanol [23].
[b] The log P value for water was used.
[c] Signal too low for detection.

zyme stabilizing effect of monohydric alcohols up to 20% v/v is a well documented phenomenon [8], as well as the denaturation of enzymes or antibodies with increasing solvent concentration [7,11]. The stability of enzymes at high concentrations of polar solvents (usually above 80%) was explained by Gladilin and Levashov [11] as the change in aggregation state; the system is obviously no longer homogeneous, but it is rather a heterogeneous one, where the protein is suspended in the organic solvent. In other words, aggregation of protein at high solvent concentration can be the reason for the high stability by self protection. Indeed, it was necessary to agitate ethanolic (>90%) antibody samples thoroughly before dilution. Vortexing of plastic tubes for 5 s was sufficient. More intensive mixing or sonication were without further effect.

With antibody B76-BF5 and the phenylurea antigen OC-2, the influence of ethanol on stability was similar to K4E7. Stability of antibodies in hexane was not tested explicitly, as enzymes and antibodies are generally stable in this non-polar solvent.

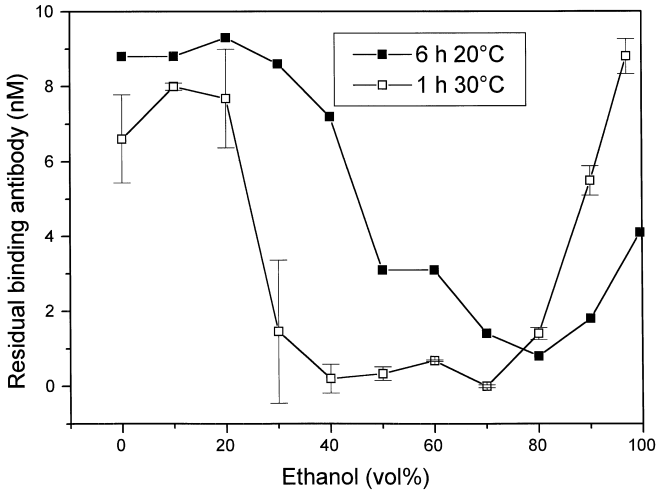

Fig. 1. Retention of antigen binding ability of antibody K4E7 after preincubation in ethanol–water mixtures up to 99.4% v/v, for 6 h at 20°C and 1 h at 30°C (standard deviation for $n = 2$). The antibody concentration was 100 nM in the preincubation mixture and 10 nM after 1/10 dilution for Biacore.

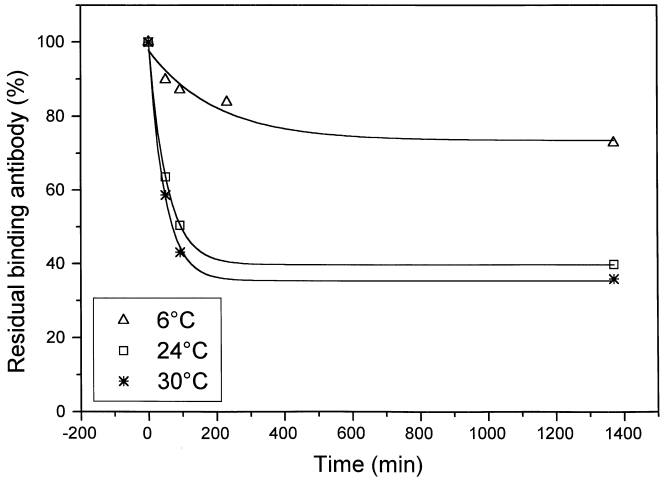

Fig. 2. Kinetics of apparent irreversible denaturation of antibody K4E7 (100 nM) in 99% ethanol at various temperatures. Activity was measured as in Fig. 1.

3.2.2. Stability of HRP in ethanol–water mixtures

Enzymes are known to be affected by organic solvents, and other labels, e.g. fluorescent dyes, may be more resistant to non-aqueous conditions. However, our intention was to use standard enzyme immunoassays for the measurement of analytes in organic solvents.

Stability curves for HRP at 20°C for 3 days are similar to the described curves for antibody K4E7. The enzyme was stable up to 20% ethanol and stability decreased at higher temperatures. A minimum stability was observed between 30 and 60% ethanol and above 90% ethanol. The minimum around 50% ethanol is consistent with published curves [3,10]. Probably, the various effects of ethanol (inactivation by removal of essential water, unfolding) and of ionic strength (destabilization in ethanol) overlap.

The stability in ethanol–water mixtures depended strongly on the phosphate buffer concentration (Fig. 3). The highest stability was observed for a phosphate concentration below 0.01 mM in the ethanol–water mixtures (data not shown). This is an important result, as in our experiments, peroxidase was diluted in ethanol from a stock solution in phosphate buffer. Using antibody K4E7, the stability dependence on phosphate ionic strength was also observed, but it was less pronounced (100% stability with 0.2 mM phosphate, 70% stability with 5–20 mM phosphate after 1 h at 20°C).

A more detailed analysis of peroxidase thermostability in solvent–water mixtures and the influence of various additives is under investigation. Also, the (de)stabilizing effect of enzyme conjugation, antibody binding or immobilization remains to be studied.

Some comments on the reversibility of denaturation are necessary here. If an enzyme is inactive in 90% ethanol, but fully active after 10-fold dilution in buffer, the activity is restored quickly and any denaturation can be regarded as reversible or transient. If the stability is not restored even after prolonged incubation in the dilution buffer, the denaturation is apparently but not necessarily irreversible. However, the aim of our experiments was not to optimize conditions for the restoration of activity, but to show what happens under the conditions used for immunoassays.

3.3. ELISA for antigens in ethanol and hexane

3.3.1. Sequential saturation ELISA

We used two modifications of an enzyme immunoassay, which differ in the incubation steps for antigen and conjugate. In the competitive assay, a mixture of antigen and conjugate was incubated with the immobilized antibody. The term sequential saturation assay was chosen to describe the assay protocol, where antigen (in the respective solvent) is bound to immobilized antibody, followed by a short pulse of

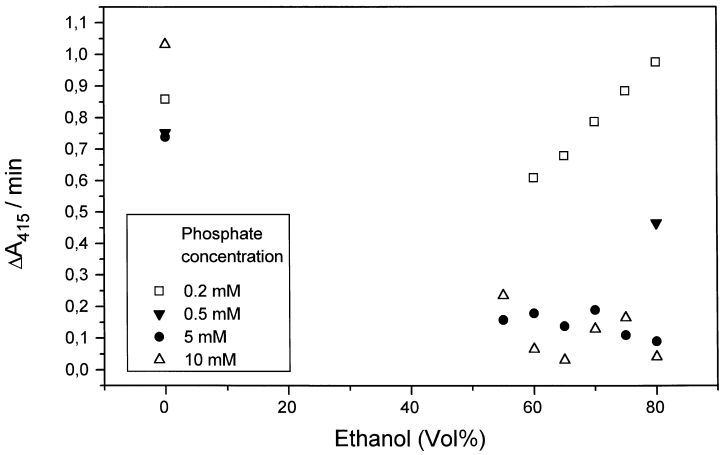

Fig. 3. Stability of HRP in aqueous mixtures up to 80% v/v ethanol, for 3 days at 20°C. The final concentration of phosphate buffer pH 7 is indicated. The preincubation mixtures were diluted 100-fold for activity measurement.

conjugate incubation. Solubility and stability of the enzyme conjugate in the solvent does not play a role in this scheme. The ELISA experiments revealed that antibodies covalently linked onto microtiter wells are stable in ethanol for 1 h at room temperature (20–25°C).

Fig. 4 shows sigmoidal plots for the triazine assay in hexane and PBST. It is evident that the background (obtained with excess atrazine) is higher in PBST than in hexane but that the midpoint IC_{50} is lower. The high background in PBST is probably due to the partial displacement of atrazine by conjugate during the conjugate incubation. The extent of displacement should depend on differences in affinity of the antibody for hapten and conjugate. The low background with hexane may be due to insufficient washing of excess atrazine in hexane before conjugate addition. Therefore, the remaining free atrazine competes probably with conjugate. The lower limit of detection (LOD) was below 0.1 nM atrazine both in hexane and in PBST. Two common definitions of LOD were considered: antigen concentration at 90% of maximum signal or antigen concentration at A_{max} minus three times of mean relative standard deviation. However, the data points in this low concentration region are not sufficient to allow accurate LOD values.

Fig. 5 shows results for phenylurea OC-2 detection in hexane, ethanol and PBST with Mab B76-BF5. In contrast to the triazine ELISA, the IC_{50} was lowest in hexane. The LOD was below 1 nM for both solvents. Results are discussed in Section 3.3.4.

3.3.2. Competitive ELISA

Re-equilibration and displacement during the 15 min of conjugate incubation led to a high background in the sequential saturation assay for atrazine in ethanol. In buffer, only 50% inhibition of the maximum signal could be achieved. Therefore, a competitive assay was performed. The maximum signal using ethanol as solvent was 1000-fold lower than with buffer at 25°C and hardly detectable. The experiment was repeated at 0°C because antibody and marker enzyme are more stable at lower temperatures. As a consequence, the difference in maximum signal was reduced to about 100-fold. Results are shown in Fig. 6. The midpoints IC_{50} were also shown to be lower at low temperatures. The poorer performance in PBST, compared with the sequential saturation assay is, at least partially, due to the high antibody coating of the wells. The midpoint IC_{50} for the assay in 99% ethanol was estimated to be 1 μM atrazine (25°C) and 300 nM (0°C), and in water, 2.8 nM (25°C) and 1.2 nM (0°C).

3.3.3. Co-solvent effects

Stock solutions of antigens were prepared in ethanol. Therefore, small amounts of ethanol were present in the antigen dilutions. We tested whether

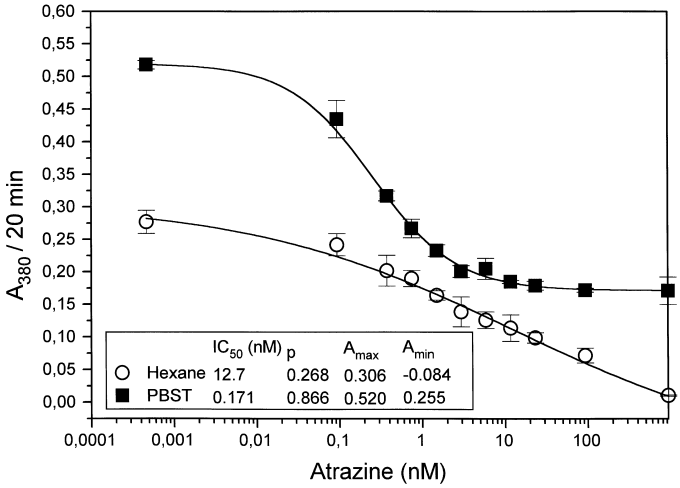

Fig. 4. Sequential saturation ELISA for atrazine in hexane and PBST with Mab K4E7 as described in Section 2.5.

low ethanol concentrations in assays with buffer, hexane or acetonitrile as diluent affected the binding of antigen to the antibody. Acetonitrile was included in this experiment in order to compare hexane with a water-miscible solvent other than ethanol. It was found that even 1% ethanol strongly reduced the OC-2 binding to immobilized antibodies in hexane (Fig. 7). Similar results were obtained when the same OC-2 concentration was presented in acetonitrile, with ethanol as the co-solvent, but no effect was found when ethanol at a concentration of up to 10 vol% in water was used. In addition to that, no difference was found for water-free and water-saturated hexane.

3.3.4. Test midpoints versus antigen solubilities

Test midpoints IC_{50} were used as an indicator of assay performance, instead of sensitivity or lower detection limit. An IC_{50} of 1 nM means that the maximum signal is 50% inhibited by 1 nM antigen. Tables

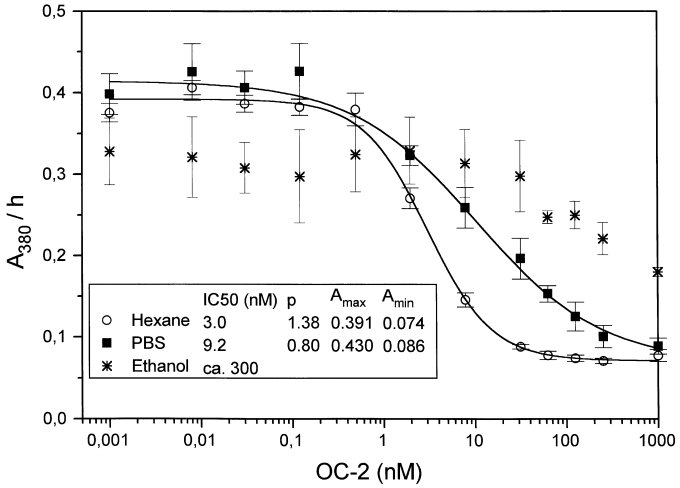

Fig. 5. Sequential saturation ELISA for OC-2 in *n*-hexane, ethanol and PBST with Mab B76-BF5 as described in Section 2.5 ($n = 2$ for ethanol).

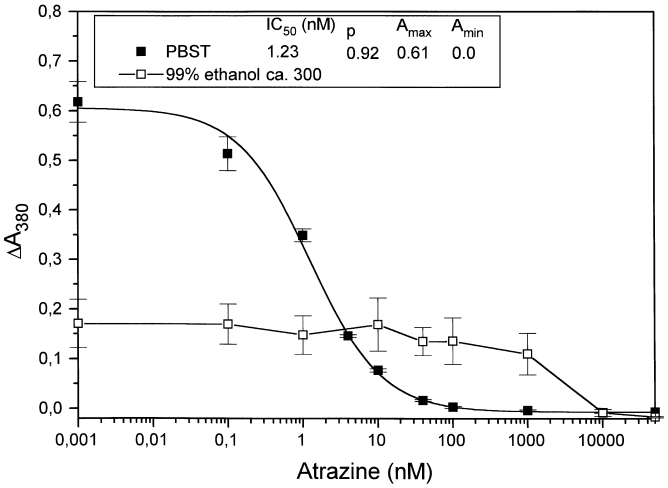

Fig. 6. Competitive ELISA for atrazine with Mab K4E7 in PBST and ethanol at 0°C. Absorbance changes were recorded within 1 min (PBST) and 33 min (ethanol).

2 and 3 summarize the test midpoints for ELISA experiments with antibody B76-BF5 and K4E7, respectively, and the solubilities of the antigens. Organic solvents affect enzyme stability and activity by dehydration and solvation not only of the substrate or antigen, but also of the enzyme or antibody. The consequences are reversible and irreversible conformational changes of the protein and changes in the free energies of binding of the interacting molecules [7]. Where water is the substrate or the product of a reaction, the thermodynamic equilibrium can be reversed [24].

The intention of this work was to find a solvent for selected antigens, which are useful both for extraction of the antigens and for ELISA in that solvent. The results show that both conditions cannot be met optimally by the same solvent. Ethanol, in which the

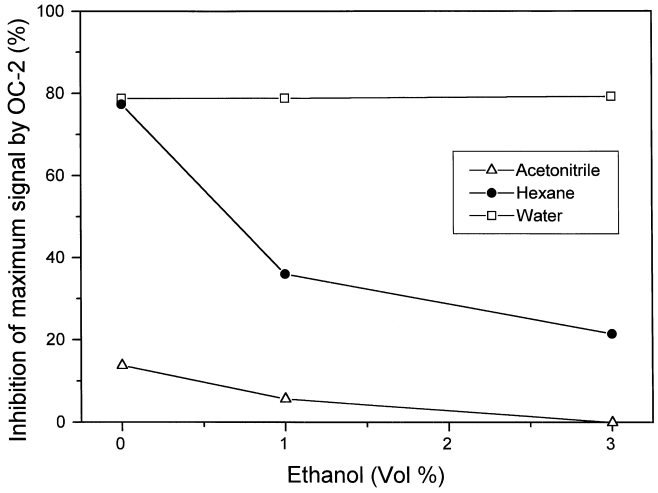

Fig. 7. Influence of co-solvent ethanol on the inhibition of maximum signal by 20 nM OC-2 in hexane, acetonitrile and water (sequential saturation ELISA). Six wells were used for each solvent without antigen and six with antigen. The mean coefficient of variation (CV) was 4.5% (without antigen) and 8.6% (with antigen: low absolute absorbance values).

Table 2
Test midpoint of sequential saturation ELISA for the detection of phenylurea OC-1 and OC-2 with Mab B76-BF5, and solubilities of the antigens

	OC-1		OC-2	
	IC_{50}	Solubility	IC_{50}	Solubility
PBS	51 nM	80 μM	9.2 nM	30 μM
Hexane	17 nM	1.5 μM	3.0 nM	3.5 μM
Ethanol	n.d.	>3.6 mM	∼300 nM	2.8 mM

antigens used in our experiments are highly soluble, increases the midpoint by a factor of about 30 for OC-2 detection, and 250–5000 for atrazine detection, depending on the assay format and conditions. On the other hand, the solubility in ethanol, compared with water, is increased by a factor of about 100 (OC-2) and 400 (atrazine).

The midpoints and solubility results for triazines in hexane followed the same trend, but the differences were less pronounced. Surprisingly, the detection of phenylurea OC-1 and OC-2 in hexane was improved, compared with buffer. However, these compounds are less soluble in hexane than in water.

The data can be interpreted in analogy to the findings of Wescott and Klibanov [25], who found inversion of substrate specificity of the enzyme subtilisin Carlsberg by solvent variation. Correspondingly, the observed affinity of antibodies (or the IC_{50} value) is a function of the solvent-to-water partition coefficients of the antigens. The driving forces are analogous to the hydrophobic effect. This means that antigens are driven to bind to the antibody especially in solvents, in which they are poorly soluble. Stangl et al. [26] described the solubilizing effects of detergents and their influence on the cross-reactivities of antibodies against triazines. They also found that pesticides which are more hydrophobic are better solubilized in the presence of detergents, and therefore, shielded to a higher degree, resulting in lower cross-reactivities.

Table 3
Test midpoint of sequential saturation ELISA for the detection of atrazine and propazine with Mab K4E7, and solubilities of the triazines

	Atrazine		Propazine	
	IC_{50}	Solubility	IC_{50}	Solubility
PBST	0.17 nM	185 μM	0.35 nM	52 μM
Hexane	12.7 nM	482 μM	4.1 nM	585 μM
Ethanol	ca. 1 μM	70 mM	n.d.	29 mM

There is also evidence from preliminary results that the IC_{50} decreased and the solubility decreased in the order hexane–heptane–octane.

Aston et al. [17] described an ELISA for a highly hydrophobic antigen, which was insoluble in water. The antigen in solvent was mixed with antibody in water, before the aqueous layer was tested in microtiter plate ELISA. In our experiments, no extraction of antigen from solvent to aqueous antibody was necessary. Other solvent based immunoassays include reversed micelles [27,28], but antigen solubility data were not described. An obviously slow phase transfer was observed by Weetall [16] with antibodies immobilized on beads, for the binding of antigen in hexane.

We have shown that it is possible to detect antigens in water miscible as well as water non-miscible solvents by a standard microtiter plate ELISA. Further experiments are planned with antibodies against more hydrophobic antigens, which are practically insoluble in water but soluble in hexane, and therefore, not detectable with standard ELISA procedures.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Innovationskolleg INK 16 A1-1.

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