Enzyme-Linked Immunosorbent Assay for Didecyldimethylammonium Chloride, a Fungicide Used by the Forest Products Industry

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A competitive enzyme-linked immunosorbent assay (ELISA), based on polyclonal antibodies, was developed to measure didecyldimethylammonium chloride (DDAC). The anti-DDAC polyclonal antibodies recognized the free DDAC in an indirect competitive ELISA. With this assay, the detection limit was 8 μg/mL, and 50% inhibition was reached at 29 μg/mL. The antibodies recognized the aliphatic chain of the DDAC molecule. They also cross-reacted with compounds having 10–12-carbon aliphatic chains, such as fatty acids, fatty alcohols, or *n*-decyl α-D-glucoside. The competitive ELISA was used to measure the amount of DDAC present in several available antisapstain products. For example, with F2A (15% DDAC), a linear relationship between the ELISA results and the theoretical values was observed in a range between 20 and 50 ppm DDAC. The measured concentrations of DDAC in other commercial products, F2, NP-1, and Bardac 2280, were in close agreement with the values reported by the manufacturer.

Keywords: *Didecyldimethylammonium chloride; antibodies; enzyme-linked immunosorbent assay*

INTRODUCTION

Canada is the world's largest market for fungicides that control stain and mold on lumber products (Smith, 1991). Since about 1970, considerable research has been done to find wood-protecting chemicals and processes to replace the traditional chlorinated phenolics, such as pentachlorophenol (PCP) and tetrachlorophenol (TCP) (Cserjesi and Roff, 1975; Byrne and Smith, 1987). In 1987 the chlorinated phenols began to be phased out by the lumber industry. Currently, products containing didecyldimethylammonium chloride (DDAC, Figure 1) account for about 95% of the Canadian sapstain control market (T. Byrne, personal communication). Such products include Bardac 2280 from Lonza Inc., NP-1 from Kop-Coat Inc., F2 from Walker Brothers, Ecobrite III from Canfor, and Timbercote II from Napier Pacific Industries Inc. DDAC, a quaternary ammonium chloride (QAC) compound, is registered with Agriculture Canada and appears to be an environmentally acceptable wood protectant. This chemical is widely used to protect freshly cut lumber against molds, wood-decaying and sapstaining fungi, and insects (Morris and Ingram, 1988; Ruddick and Ingram, 1987; Tsunoda and Nishimoto, 1987; Wallace, 1986; Linderborg, 1984; Sundman, 1984; Howick et al., 1983; Butcher and Greaves, 1982; Hulme and Thomas, 1983; Preston and Chittenden, 1982; Butcher et al., 1977; Butcher and Drysdale, 1978; Cross, 1979).

DDAC does not occur in nature. It is relatively stable to light and hydrolysis and resistant to microbial degradation (Henderson, 1992). Its toxicity, oral LD_{50} = 450 mg/kg for mouse, is much lower than for PCPs, LD_{50} = 70 mg/kg (Anonymous, 1984). DDAC and other QACs are well-known for their activity against bacteria, fungi, and algae (Gosselin et al., 1984). They have been widely used as general microbiocides in disinfectants, sanitizers, eyedrops, and paint (Kop-Coat Inc., 1988). They are also used as surfactants in lubricants, antistatic agents, fabric softeners, hair conditioners, and dye additives.

Traditionally, DDAC has been detected by wet chemistry and colorimetric assays. However, these assays are insensitive, are often not specific, and are subject to interference, especially for wood and environmental analysis (Lonza Inc., 1988). Using conventional flame ionization, nitrogen selective, and mass spectrophotometric detectors, DDAC has been analyzed by GC taking advantage of the pyrolysis occurring on the column during the process (Crowther and Fairchild, 1991; Goetz et al., 1985; Metcalfe, 1963). To minimize the problems caused by the surfactant nature of DDAC and the pyrolysis of the quaternary ammonium center, Crowther and Fairchild (1991) used a deuterated internal standard. Several HPLC methods have been reported for the analysis of DDAC with reasonable accuracy (Daniels, 1992; Conboy et al., 1990; Matthijs and de Henau, 1987; Wee and Kennedy, 1982). One of them uses evaporative light scattering detection and was used for the Antisapstain Quality Assurance Program developed by Forintek Canada Corp., Vancouver, BC. This method can measure as low as 5 μg of DDAC. None of the above methods can be performed directly on wood. All of them demand an initial extraction step, and most of them also require a partial purification of the extract. These methods are time-consuming, expensive, and not adequate for handling a large number of samples.

Immunoassays may offer an alternative to, or could complement, traditional analytical methods. The acceptability of immunoassays is increasing for pesticide and other environmental analyses (Marco et al., 1993; Abouzied et al., 1993; Harrison et al., 1991a; Giersch and Hock, 1990; Jung et al., 1989; Li et al., 1989;

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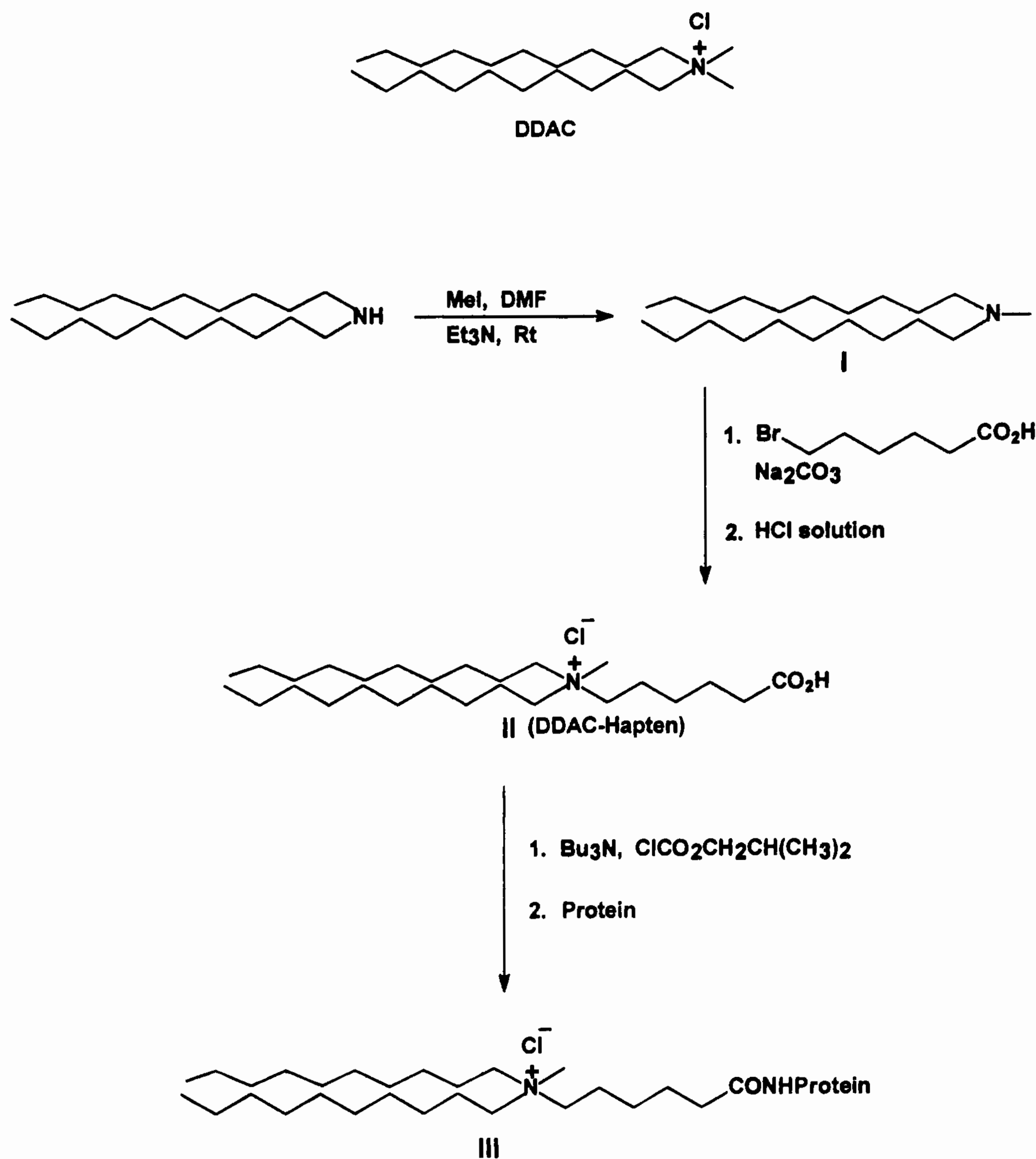

Figure 1. Synthesis of the DDAC–hapten, immunogen, and coating antigen. The carrier protein for the immunogen was KLH; for the coating antigen, it was BSA.

Hammock and Mumma, 1980). In this paper we describe the development of an immunoassay for the detection of DDAC using anti-DDAC polyclonal antibodies. The work includes designing and synthesizing the hapten, generating and characterizing the antibodies, and using the assay for measuring DDAC in commercial antisapstain fungicides.

MATERIALS AND METHODS

Reagents. Bardac 2280 was obtained from Lonza Inc. Pure DDAC (98%), F2, F2A, and NP-1 were from Forintek. Fatty acids, bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), Freund's complete and incomplete adjuvants, carbonate/bicarbonate buffer capsules, *o*-phenylenediamine dihydrochloride (OPD) tablets, *n*-decyl α-D-glucoside, and Tween 80 were purchased from Sigma. Isobutyl chloroformate, didecylamine, methyl iodide, tri-*n*-butylamine, 6-bromohexanoic acid, Et_4NCl, and trimethylammonium hydrochloride were purchased from Aldrich. The anti-rabbit IgG–horseradish peroxidase was obtained from Caltag Laboratories (San Francisco, CA). Bacto dehydrated skim milk was obtained from Difco Laboratories (Detroit, MI). All solvents were of HPLC grade, and the deionized water was purified by the NANOpure ultrapure water system (Barnstead-Themolyne).

Equipment. Microtitration plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA) and a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA) were used for the ELISA. A Perkin-Elmer 1600 Series FTIR spectrometer and a Bruker WH-400 NMR spectrometer were used for the analysis of the products synthesized.

Immunogen Design and Hapten Synthesis. Since DDAC and related compounds do not have a suitable functional group to be conjugated with carrier proteins, a new DDAC hapten with a carboxylic group was synthesized. A six-carbon spacer arm was introduced between the nitrogen and the carboxylic group to avoid steric hindrance or alteration of the DDAC structure. The (ω-carboxyhexanyl)didecylmethylammonium chloride was synthesized as shown in Figure 1.

Didecylmethylamine (I). Didecylamine (10 g, 33.6 mmol) and methyl iodide (5.0 g, 35.3 mmol) were dissolved in 100 mL of dried DMF, then 6.7 g (35.3 mmol) of tri-*n*-butylamine was added, and the solution was stirred at room temperature for 3 days. After filtration, the solvent was removed, and the

residue was redissolved in 100 mL of ether and then washed with 0.5% of NaOH (2 × 20 mL). The ether solution was dried over anhydrous potassium carbonate. The ether and the didecylmethylamine were removed by distillation under reduced pressure. The yield was 67%: ^{1}H NMR ($CDCl_3$) δ 0.91 (t, 6H), 1.22–1.64 (m, 32H), 2.12 (s, 3H), 2.60 (t, 4H).

*(ω-Carboxyhexanyl)didecylmethylammonium Chloride (DDAC–Hapten, **II**).* A mixture of 2 g (0.64 mmol) of didecylmethylamine (**I**), 1.51 g (0.77 mmol) of 6-bromohexanoic acid, and 0.25 g (1.81 mmol) of potassium carbonate in 100 mL of dried dioxane was refluxed for 72 h. The residue obtained after filtration and removal of the solvent was dissolved in 50 mL of water. Hydrochloric acid (37%) was added until the pH reached a value of 2. The above solution was extracted with dichloromethane (3 × 50 mL), and the combined extracts were dried over anhydrous potassium carbonate. The solvent was removed, and an oily product identified by white silver chloride precipitation as (ω-carboxyhexanyl)didecylmethylammonium chloride was obtained with a 60% yield. The nature of the final product was confirmed by ^{1}H NMR ($CDCl_3$): δ 0.80 (t, 6H), 1.19–1.80 (m, 38H), 2.13 (s, 3H), 2.40 (t, 6H), 3.15 (t, 2H). Its carboxylic functionality was also supported by FTIR: 3460 (br, w) and 1725 (s).

Hapten Coupling. The DDAC–hapten (26.3 mg, 0.062 mmol) was dissolved in 1 mL of dry dioxane. Tri-*n*-butylamine (17.9 μL, 0.074 mmol) and isobutyl chloroformate (9.7 μL, 0.074 mmol) were added, and the resulting mixture was stirred at room temperature for 30 min. This mixture was added dropwise to 15 mL of solution, containing 50 mg of proteins in 0.2 M borate buffer, pH 8.7. The solution was stirred gently overnight at room temperature. Bovine serum albumin (BSA) was used to prepare the coating antigen and keyhole limpet (KLH) to prepare the immunizing antigen. The conjugates were dialyzed for 24 h against water and then lyophilized.

Producing and Screening the Polyclonal Antibodies. Four New Zealand female white rabbits weighing between 2 and 4 kg were immunized intradermally. Each rabbit received 0.5 mL of an emulsion (1:1) containing 0.25 mg of DDAC–hapten–KLH in saline and complete Freund's adjuvant. The rabbits were boosted three times with a 2-week resting interval, using the same amount of antigen emulsified in incomplete Freund's adjuvant. The titer of the anti-DDAC serum was determined by an indirect enzyme-linked immunosorbent assay (ELISA). When a high titer was reached, the rabbits were bled and the serum was separated by centrifugation and stored at −70 °C.

Indirect ELISA and Competitive Inhibition ELISA. Microtitration plates were coated with DDAC–hapten–BSA in $NaHCO_3$, pH 9.6, and dried overnight at 37 °C. The plates were washed four times with PBS and blocked with 200 μL/well of 2% milk in PBS for 1 h at 37 °C and then washed four times with PBS. Diluted DDAC polyclonal serum in 0.1% milk in PBS was added to the wells and incubated at 37 °C for 2 h. Then the plate was washed four times with PBS, and a secondary antibody-enzyme conjugate, the anti-rabbit IgG–horseradish peroxidase diluted 1:3000 into 0.1% milk in PBS, was added. Both the diluted serum and the antibody–enzyme conjugate were applied at 100 μL/well. The enzymatic reaction was carried out at room temperature, in the dark, using the SIGMA *FAST* *o*-phenylenediamine dihydrochloride (OPD) as substrate. The reaction was stopped after 30 min with 50 μL of 2.5 M H_2SO_4, and the absorbance was measured at 490 nm.

A competitive inhibition ELISA was used for assessing the specificity of the antibodies to free DDAC and the cross-reactivity of related compounds. Stock solutions of 1000 ppm DDAC or related chemicals were prepared in methanol. Appropriate concentrations of the chemical mixed with the DDAC polyclonal antiserum diluted 2000 times into 0.1% milk in PBS were then added to a plate which had been coated with DDAC–hapten–BSA and blocked with 2% milk in PBS. The rest was carried out as described for the indirect ELISA procedure.

RESULTS AND DISCUSSION

Hapten Design. As immunoassay specificity and sensitivity are determined primarily by the antibody produced in response to the immunogen, hapten design and synthesis are critical steps in the development of immunoassays to chemicals (Harrison et al., 1991). Among the quaternary ammonium compounds, DDAC has the highest antifungal activity. This activity is mainly related to the two decyl (C_{10}) aliphatic chains (Preston, 1983). Consequently, the ideal hapten should retain the C_{10} aliphatic chains and the quaternary ammonium nitrogen center and should have a functional group to which proteins can be conjugated. We designed a DDAC–hapten with the above characteristics (Figure 1, **II**). In addition, the carboxylic group which was used for conjugating the protein was linked to the quaternary ammonium nitrogen center through a spacer arm. The effects of the length of a spacer arm on the sensitivity and specificity of the antibodies produced against pesticides have been well documented by Harrison et al. (1991b). A spacer arm that is neither too long nor too short maximizes the exposure of the analyte for antibody production and increases the assay sensitivity. Consequently, we linked a five-carbon alkyl spacer arm [$(CH_2)_5$] with a carboxylic group to the didecylmethylammonium moiety.

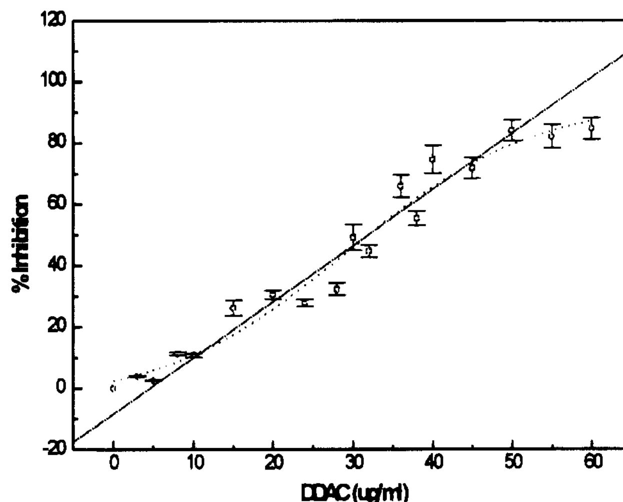

Figure 2. Standard inhibition curve for the anti-DDAC polyclonal antibodies using free DDAC. The indirect competitive ELISA was done using a serum dilution of 1:2000 and a coating antigen concentration of 100 ng/well (see Materials and Methods). Percent inhibition was calculated $100[1 - (A_{490}^{(x)}/A_{490}^{(ctl)})]$, where x and ctl refer to the unknown and control, respectively. The linear range was 10–50 μg/mL, with a correlation coefficient of 0.96. The experiment was repeated twice, with four replicates per value in each experiment.

Characterizing the Anti-DDAC Polyclonal Antibodies. *Titer and Inhibition Curve.* Since DDAC–hapten–KLH was the immunogen, the antisera were screened by indirect ELISA(s) using DDAC–hapten–BSA as coating antigen. An optical density reading of 1.1 was obtained with an antiserum dilution of 1:2000 and a coating antigen concentration of 100 ng/well. The end point titration was reached for an antiserum dilution of 1:32000. Increasing the coating antigen above 100 ng/well did not significantly increase the overall reaction. Antisera from all rabbits showed similar profiles. To be in the linear part of the response curve, which required an absorbance of about 1, a coating antigen concentration of 100 ng/well and an antiserum dilution of 1:2000 were used for the rest of the experimental work. The specificity of the antiserum against free DDAC was determined in an indirect competitive ELISA. A typical inhibition curve is shown with DDAC concentrations ranging from 0 to 60 ppm

Table 1. Solvent Effect on the Immunoreactivity of the Anti-DDAC Polyclonal Antibodies: Percent Residual Activity vs Solvent Concentration

	solvent concn								
solvent	0.25%	0.5%	1%	2%	3%	4%	5%	6%	8%
DMSO	103	88	84	82	56				
ethanol	103	94	84	88	59				
acetone	92	73	64	52	42				
methanol			98	98	88	83	80	71	68

(Figure 2). The percentage of inhibition was calculated as $100[1 - (A/A_0)]$, where A_0 was the absorbance with no DDAC present and A was the absorbance in the presence of DDAC. The I_{50} value for this assay was 29 μg/mL and the detection limit 8 μg/mL. The detection limit was defined as the lowest concentration of DDAC giving an absorbance value separated from A_0 by 3s, where s was the standard deviation of the blank absorbance value (Fleeker, 1987).

Effects of Solvents on the Antibody. In the cross-reactivity study (below) we had to dissolve compounds related to DDAC in solvents similar to those often present in commercial products containing DDAC. We determined the effect of different solvents, ethanol, DMSO, methanol, and acetone, on the activity of the DDAC antibodies (Table 1). The DDAC antibodies retained their immunoreactivity in the presence of 3% methanol. However, ethanol, DMSO, and acetone at concentrations equal to or greater than 1% reduced the reactivity of the serum by 16–36%. Similar effects on the immunoreactivity of antibodies have been reported for low concentrations of organic solvents (Bekheit et al., 1993; Wittmann and Hock, 1993; Harrison et al., 1991a). However, other researchers have claimed that immunoassays can tolerate high concentration of solvents (up to 10%), though in some cases it was necessary to incorporate the solvent in the standard curve to take into account minor effects on the final reaction (Krämer et al., 1994; Lucas et al., 1993). Consequently, we avoided using acetone and used the other organic solvents only in trace amounts, when necessary in the ELISA.

Specificity of the Polyclonal Antibodies. It is well-known that antibodies produced from a given immunogen will recognize a compound's different moieties to different degrees. The chemical structures of DDAC and DDAC–hapten can be divided into four types of possible recognizable moieties, i.e., the long aliphatic chain, the methyl group, the quaternary ammonium cation, and the nitrogen center. A series of compounds with structures similar to the different parts of DDAC was tested for their cross-reactivities with the anti-DDAC polyclonal antibodies (Table 2). The chemicals tested were grouped into four categories according to their similarities. The results from an indirect competitive ELISA indicated that the DDAC polyclonal antibodies did not react significantly with either the short-chain quaternary ammonium cation or the nitrogen center. The cross-reactivity varied with different types of compounds having aliphatic chains, i.e., quaternary ammonium salts, fatty acids, fatty alcohols, and alkyl glucosides. None of the very long (Table 2, entries 10,

Table 2. Percentage Cross-Reactivity of the Anti-DDAC Polyclonal Serum with Chemicals Related to DDAC[a]

entry	chemical (30 ppm)	structure	cross-reactivity (%)
1	DDAC		100 ± 1
		Short-Chain Quaternary Ammonium Compounds	
2	Me_3NHCl	CH_3, CH_3, N^+, Cl^-, CH_3, H	1.6 ± 0.7
3	Me_2NH_2Cl	CH_3, H, N^+, Cl^-, CH_3, H	4.5 ± 0.7
4	choline chloride	CH_3, H, N^+, Cl^-, CH_3, OH	9.0 ± 1.0
5	Et_4NCl	Et, Et, N^+, Cl^-, Et, Et	3.0 ± 0.1
		Fatty Acids	
6	acetic acid (C2:0)	CO_2H	0
7	octanoic acid (C8:0)	CO_2H	10.5 ± 0.4
8	decanoic acid (C10:0)	CO_2H	37 ± 3
9	undecanoic acid (C11:0)	CO_2H	54 ± 5
10	stearic acid (C18:0)	CO_2H	0
		Fatty Alcohols	
11	1-octanol (C8:0)	CH_2OH	22 ± 3
12	1-decanol (C10:0)	CH_2OH	37 ± 5
13	1-undecanol (C11:0)	CH_2OH	52 ± 6
14	1-steary alcohol (C18:0)	CH_2OH	9 ± 1
		Others	
15	*n*-decyl D-glucopyranoside	with a 10-carbon aliphatic chain	66 ± 6
16	Tween 80	with an 18-carbon aliphatic chain	0

[a] Coating antigen: DDAC–BSA, 100 ng/well; polyclonal serum dilution 1:2000.

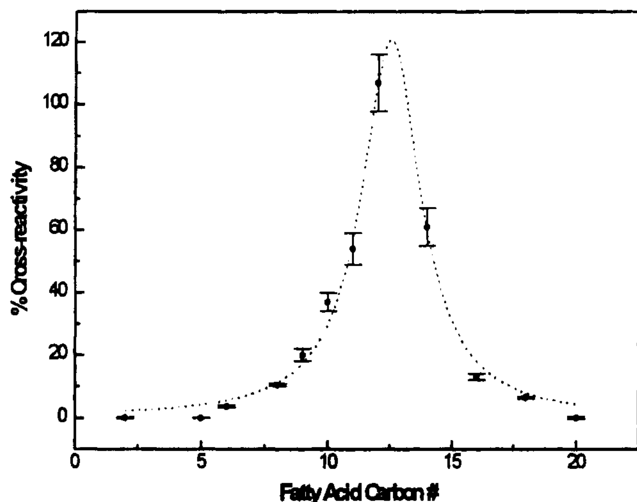

Figure 3. Cross-reactivity of the anti-DDAC antibodies with different fatty acids. The fatty acids concentration used was 30 μg/mL. The indirect competitive ELISA was done using a serum dilution of 1:2000 and a coating antigen concentration of 100 ng/well (see Materials and Methods). The percentage cross-reactivity (CR) for each fatty acid, x, was calculated as 100[% inhibition(x)/% inhibition([DDAC] = 30 μg/mL)]. The experiment was repeated three times with seven replicate wells per fatty acid per assay. The error bars represent $\%CR_{(x)} \pm SE_{(x)}$.

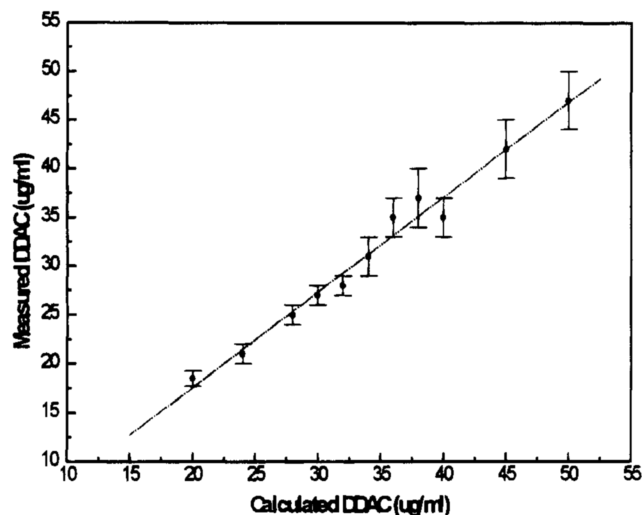

Figure 4. Correlation curve between calculated DDAC and measured DDAC by ELISA for a product, F2A. The indirect competitive ELISA was done using a serum dilution of 1:2000 and a coating antigen concentration of 100 ng/well (see Materials and Methods). The error bars represent standard error of the mean. The slope was 0.97 ± 0.01, and the correlation coefficient was 0.99.

Table 3. Percentage of DDAC Recovered from Samples Spiked with Known Amounts of DDAC

spiked DDAC concn (μg/mL)	measd DDAC concn (μg/mL)	recovery (%)
0	−6 ± 3	
12	13 ± 4	108
18	15 ± 5	83
23	22.0 ± 0.4	96
28	28 ± 3	100
30	29 ± 2	97
32	32.3 ± 0.3	101
38	40.0 ± 0.9	105
42	44 ± 2	105
48	47.25 ± 0.05	98

14, and 16) or short aliphatic chain compounds (Table 2, entries 2−6) were recognized by the antibodies at concentrations below 50 ppm. Only compounds with straight aliphatic chain residues containing 10−12 carbons strongly cross-reacted with the serum and competed with the coating antigen in the ELISA. For example, compounds with 11- or 10-carbon aliphatic chains, such as undecanoic acid, 1-undecanol, and *n*-decyl α-D glucoside, showed 54, 52, and 66% cross-reactivity, respectively. The C_{10} aliphatic chain of the designed immunogen, because of its size, might be more visible or more immunogenic than the methyl group or the quaternary ammonium cation.

The cross-reactivity of the antiserum with other fatty acids was further examined (Figure 3). Maximum cross-reactivity was observed with the C_{12} fatty acid, and the cross-reactivity decreased as the fatty acid carbon number differed from C_{12}. Both undecanoic acid (C_{11}) and tetradecanoic acid (C_{14}) showed 56 and 60% cross-reactivity, respectively; octanoic acid (C_8) and palmitic acid (C_{16}) showed 20% cross-reactivity. No cross-reactivity was observed with fatty acids containing fewer than 7 or more than 18 carbons. A similar pattern was observed for the fatty alochols. Why was fatty acid C_{12} more reactive than C_{10}? The DDAC−hapten−KLH immunogen has two 10-carbon chains and a cationic head, as has free DDAC. However, fatty acids have an anionic head (carboxylic), which might interfere with binding to the antibody. The extra carbon atom of the C_{12} fatty acid could act as a short spacer that removed the anionic head from the binding site, attenuating the interference of the electronic repulsion, and giving the C_{12} fatty acid a better binding capacity to the antibody than the C_{10}.

Determining the DDAC Content of Various Antisapstain Products Using the Polyclonal Antiserum in a Competitive ELISA. Each ELISA plate used for a DDAC determination via a competitive assay included samples for a standard curve and one or more unknowns. Standard curves were generated using 98% pure DDAC and were linear for DDAC concentrations between 10 and 50 μg/mL. The linear regression equation for each plate's standard curve was used to calculate the DDAC concentrations of the unknowns on that plate.

First, a set of distilled water samples was spiked with pure DDAC (98%) at concentrations ranging from 12 to 48 μg/mL. The results in Table 3 show that recovery values were between 83 and 108% of the spiked values. Next we determined whether the antiserum to DDAC was able to measure DDAC in different fungicide products. The antisapstain product F2A, containing 15% DDAC, was tested at assay concentrations ranging from 20 to 50 ppm DDAC. Figure 4 shows the correlation between the measured and the theoretical DDAC concentrations. A straight line with a slope of 0.97 ± 0.01 and a correlation coefficient of 0.99 was obtained. Then, using the same method, we examined the DDAC contents of other commercial products. The results are listed in Table 4. In general, the results obtained by the competitive ELISA were within 7% of the theoretical values reported by the commercial company.

Since the commercial products contain DDAC and other ingredients such as borax (F2), ethanol (Bardac 2280), and 3-iodo-2-propynyl butyl carbamate (NP-1), the effect of these components on the immunoassay was assessed. The concentration of the chemical tested was similar to the concentration present in the commercial products when measured by the immunoassay. Borax at concentrations up to 100 μg/mL, and ethanol at

Table 4. Analysis of DDAC in Antisapstain Products Using the Anti-DDAC Serum in a Competitive ELISA

product (30 μg/mL DDAC)[a]	$DDAC_{measd}$ (μg/mL)
DDAC (pure)	30 ± 3
NP-1	31 ± 3
F2A	28 ± 5
F2	29 ± 3
Bardac 2280	31 ± 3

[a] The concentration was calculated from the reported DDAC in the antisapstain products. All but F2A are commercial products.

concentration less than 1%, had no effect on the immunoassay. 3-Iodo-2-propynyl butyl carbamate (IPBC) alone at a concentration equivalent to the commercial product (5.9 μg/mL) affected the overall reaction by 30%. However, when 1−6 μg/mL IPBC was mixed with 10−50 μg/mL of DDAC (98%), no interference was observed. This confirmed that, when present in the formulation, these chemicals did not interfere with the measurement of the active ingredient DDAC.

Since the polyclonal antibodies recognized only the two long aliphatic chains of DDAC, such antibodies could be used to measure DDAC in commercial antifungal products. The work reported here suggests that such an immunoassay could be carried out directly on the original product, without requiring any extraction or purification steps.

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