Improved Methods for Conjugating Selected Mycotoxins to Carrier Proteins and Dextran for Immunoassays

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Simplified protein-coupling chemistries were utilized for the mycotoxins, T-2 toxin (T-2), ochratoxin A (OA), citrinin (CTN), and aflatoxin B_1 (AFB_1). Iodoacetate (IAc) and 1,1′-carbonyldiimidazole (CDI) were found to be excellent activators of hydroxyl and carboxyl groups of these mycotoxins. Iodoacetate reacted with the C-3 hydroxyl groups of T-2 toxin to form methylcarboxylated T-2 (T-2-metCOOH) containing a two-carbon spacer and a stable ether-based bond. 1,1′-Carbonyldiimidazole directly activated the carboxyl groups of OA, T-2-metCOOH, the C-3 hydroxyl group of T-2, succinic anhydride modified T-2, the C-8 hydroxyl group of citrinin, and carboxymethoxylamine hemihydrochloride modified AFB_1. The resulting acylimidazole and imidazole carbamate intermediates reacted readily with the primary amine groups in proteins and ethylenediamine modified dextran to form stable amide or carbamate linkages. These coupling chemistries minimized the formation of undesired immunodeterminants in the conjugates. Toxin–protein coupling ratios could be readily controlled by varying the amount of toxin relative to that of protein in the CDI coupling reaction. These results demonstrate that IAc and CDI are excellent and versatile chemical couplers of hydroxyl- and carboxyl-containing mycotoxins and complement other chemistries in mycotoxin ELISA development.

Keywords: *T-2 toxin; ochratoxin A; citrinin; aflatoxin B_1; conjugation; 1,1′-carbonyldiimidazole; iodoacetate; ELISA; aminated Dextran*

INTRODUCTION

The widespread occurrences of mycotoxins in foods and feeds pose a serious health threat to humans and animals; the most toxic of these mycotoxins are T-2 toxin (T-2), ochratoxin A (OA), aflatoxin B_1 (AFB_1), and possibly citrinin (CTN) (CAST,1989). Immuno-based assays have been developed for the detection and quantification of mycotoxins (Pestka, 1988; Chu, 1990, 1992; Candlish, 1991; Wilkinson et al., 1992). Mycotoxins by themselves are incapable of eliciting immune responses due to their small size but can be rendered immunogenic by covalent coupling to large molecular weight carrier proteins (Harlow and Lane, 1988). Mycotoxins such as T-2, OA, CTN, and AFB_1 have different functional groups (Figure 1), and as a result several different coupling chemistries have been utilized. Some mycotoxins such as OA contain an active carboxyl group, and others such as T-2 and AFB_1 require the introduction of a group having a carboxyl functionality. These carboxyl groups can then be activated and covalently coupled to amine groups in proteins to form stable amide linkages (Wilkinson et al., 1992; Candlish, 1991). Several amide-bond formation reactions have been employed including those that make use of the *N*-hydroxysuccinimide activated esters (NHS), water-soluble carbodiimides (WSC), and mixed anhydrides (MA). Several problems have been encountered with the conventional MA, WSC, and hemisuccinate chemistries,

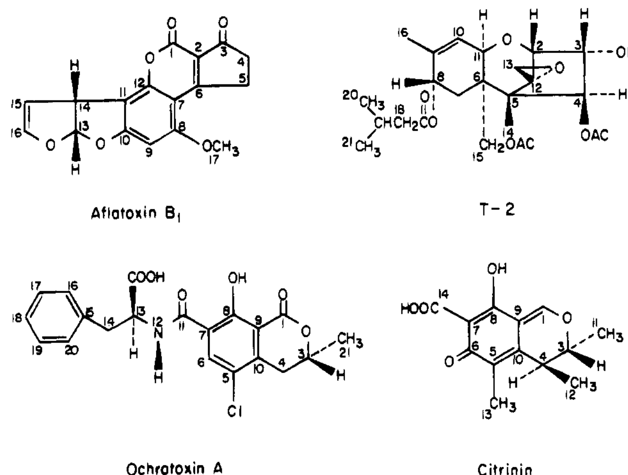

Figure 1. Structures of T-2 toxin (T-2), ochratoxin A, citrinin, and aflatoxin B_1.

specifically, formation of cross-reacting and immunogenic side-reaction products and interfering carrier–toxin bridging groups (Gendloff et al., 1986; Clarke et al., 1993; Pestka, 1988). These interferences manifested themselves as high background absorbances and reduced sensitivities in the immunoassays. It would therefore be useful to have a generalized coupling strategy that minimizes the formation of nontoxin determinants and cross-reacting bridging groups. 1,1′-Carbonyldiimidazole (CDI) is a multifunctional coupling reagent that can form noncharged and zero-length amide and carbamate bonds. This reagent has been used for hapten conjugation but is more extensively used in solid-phase matrix activation (Erlanger, 1980; Hermanson et al., 1992; Wong, 1991). The carboxyl and hydroxyl groups of target molecules are readily converted by CDI to an acylimidazole or imidazole carbam-

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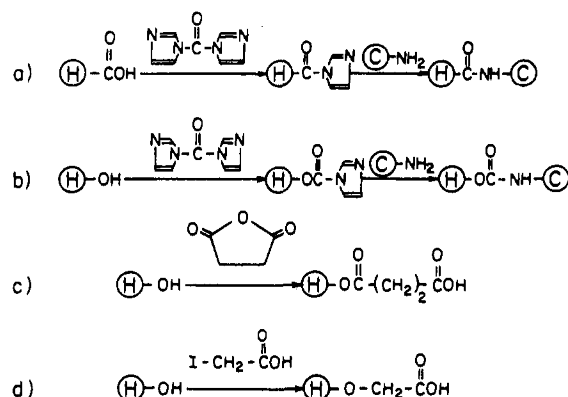

Figure 2. Covalent coupling of carboxyl- and hydroxyl-containing haptens (H) to an amine-containing carrier (C). The reactions involve the interaction of CDI with the hydroxyl group of a carboxylic acid (a) or an alcohol (b) to form acylimidazole or imidazole carbamate intermediates, respectively. These intermediates are then replaced by nucleophiles such as amines to form a stable peptide linkage. Activation of the hydroxyl group of H with succinic anhydride (c) forms a hemisuccinate and with iodoacetate (d) forms a carboxymethyl linkage or an ether-based bond.

ate intermediate, respectively. These intermediates are then replaced with nucleophiles such as amines to form stable covalent linkages (Figure 2a,b). The CDI reaction should therefore complement other carboxyl and hydroxyl mycotoxin coupling chemistries. Iodoacetate (IAc) is a reagent that is primarily used in the carboxymethylation of free thiols and amine groups of proteins (Wong, 1991) but can also directly activate hydroxyl groups (Henderson et al., 1992). The nucleophilic groups on the proteins or haptens displace the iodine of IAc and thereby become carboxylated, forming a two-carbon spacer containing an ether linkage (Figure 2d). This type of linkage also tends to be much more resistant to hydrolysis than the ester-based linkages obtained with the cyclic anhydrides such as succinic anhydride (Figure 2c). This study describes two alternative reaction chemistries suitable for the conjugation of hydroxyl- and carboxyl-containing mycotoxins. Optimal conditions for the preparation of T-2, OA, CTN, and AFB_1 protein conjugates and for the amination of dextran suitable for the conjugation of T-2 and OA are described. These reactions are simple, efficient, and controllable and produce stable and antigenic conjugates that are suitable for cELISA development. They appear to be superior to other commonly used mycotoxin coupling reactions.

MATERIALS AND METHODS

Materials. Ochratoxin A was isolated from a liquid culture of *Aspergillus ochraceus* Whilhelm (NRRL 3174) (Sreemannarayana *et al.*, 1988). Ochratoxin α (Oα) was prepared by acid hydrolysis of OA (Doster and Sinnhuber, 1972). Bovine serum albumin (BSA), ovalbumin (OV), CDI, IAc, Freund's complete and incomplete adjuvants, dextran (MW 500 000–1 000 000), ethylenediamine, phenylethylamine, *p*-nitrophenyl phosphate, diethanolamine, succinic anhydride, CTN, AFB_1, T-2, rabbit anti-mouse IgG and goat anti-rabbit IgG coupled to alkaline phosphatase, and rabbit and mouse anti-T-2 and anti-AFB_1 antibody were obtained from Sigma Chemical Co., St. Louis, MO. Mouse anti-OA (OTA7) was generously donated by Dr. Y. Ueno, Science University of Tokyo, Ichigaya, Tokyo. Laying hen and rabbit anti-OA antisera were prepared as previously described (Clarke et al., 1993, 1994). Commercial rabbit anti-chicken IgG conjugated to alkaline phosphatase was from Jackson Laboratories, West Grove, PA, and *O*-carboxymethoxylamine hemihydrochloride was from Aldrich Chemical Co., Milwaukee, WI. Dry sodium metal was from J. T. Baker Chemical Co., Phillipsburg, NJ. Microtiter plates (Falcon 3911, microtest III) were from Becton Dickinson Labware, Oxnard, CA, and KC-18 reversed-phase TLC plates were from Whatman, Clifton, NJ. All solvents and reagents used were of analytical grade.

Preparation of Immunogens and Plate Coating Antigens. *Direct Activation of OA and Covalent Coupling to Carrier Antigens.* The carboxyl functional group of OA was activated directly with CDI and allowed to react with the lysine groups on the protein to form an amide bond. In brief, 1 mg of OA and 1.5 mg of CDI were dissolved in 100 μL of acetone, mixed, and allowed to react for 10 min at 25 °C in the dark with constant stirring. The activated OA mixture was added slowly and dropwise to a continuously stirred solution of BSA or OV (10 mg in 1 mL of 0.1 M Na_2CO_3, pH 9.6). The solution was allowed to react for 2 h at 25 °C in the dark with continuous stirring followed by extensive dialysis with 0.05 M carbonate buffer (pH 9.6) to remove any uncoupled OA and subsequently distilled water to remove carbonate salts. Alternatively, the free toxin could be removed by repeated precipitation of the conjugate with cold acetone (−20 °C). In brief, the conjugates (10 mg mL^{-1}) upon completion of the reaction were transferred to centrifuge tubes and precipitated with excess volumes of cold acetone. The ratio of acetone to enzyme solution was usually 2:1 (v/v), with the maximum concentration of acetone being 80% (v/v). The mixture was centrifuged for 10 min at 1500*g* and the supernatant discarded. The pellet was redissolved with phosphate-buffered saline (pH 7.2) and reprecipitated with acetone. The conjugates (BSA–OA, OV–OA) following cleanup were collected, diluted with pH 7.2 phosphate-buffered saline (Harlow and Lane, 1988) to a concentration of 1 mg mL^{-1}, and stored at −20 °C. The extent of OA conjugation to protein was qualitatively and quantitatively assessed using thin-layer chromatography (TLC) and UV–vis spectrophotometry, respectively. The reaction mixture (5 μL) was applied to a KC-18, reversed-phase TLC plate. Standard OA and BSA (5 μL) were added separately and in combinations to the TLC plate as negative controls. Samples after spotting were air-dried, and the TLC plates were developed with a mobile phase consisting of acetone and methanol (1:1). The protein-conjugated toxin remained at the point of sample application (R_f value 0.0), and the unbound OA migrated at or near the solvent front (R_f value 0.8–0.9). No OA (blue fluorescence under UV light) was observed at the origin in the case of the negative controls, whereas an intense blue fluorescence was observed at the point of application (R_f value 0.0) with the successful OA–protein conjugates. Free OA was also observed in the conjugate preparations prior to cleanup, suggesting hydrolysis of the activated intermediates by water. The coupling ratios of OA to BSA were determined by spectrophotometric methods. The protein concentration was determined according to the Bradford method (Bradford, 1976), while the amount of OA present in the conjugate was determined by the change in absorbency at 380 nm (pH 8.5, ϵ = 7800). The presence of unbound toxin caused substantial interference with the UV spectrophotometric analysis; thus, useful results could only be obtained after extensive cleanup of the conjugates.

Amination of Dextran and Covalent Coupling of OA. Dextran was covalently modified with ethylenediamine prior to OA coupling (aminodex). In general, CDI (20 mg) was added to 10 mg of dextran in 5 mL of anhydrous acetone. The mixture was shaken and allowed to react at 25 °C for 12 h. The excess of soluble CDI following completion of the reaction was removed by filtration through a glass Pasteur pipet packed with glass wool. The activated dextran was resuspended in 5 mL of anhydrous chloroform. Ethylenediamine (5 mL) was then added to the activated dextran, and the reaction was allowed to proceed for 2 h at 0 °C. The aminated dextran (aminodex) was cleaned by repeated precipitation with cold acetone (−20 °C), dissolved in 0.1 M Na_2CO_3 (pH 9.6), and conjugated to OA as described above (aminodex–OA).

Direct Activation of T-2 Toxin and Covalent Coupling to Carrier Antigens. The hydroxyl group at position C-3 of T-2 was directly activated and coupled to protein or aminodex with CDI. In brief, T-2 (1 mg) was disolved in 100 μL of dimethyl sulfoxide (DMSO) and reacted with 1.5 mg of CDI at 25 °C for 5 min. The mixture was then added to 10 mg of protein (BSA) or 20 mg of aminodex in 1 mL of 0.1 M Na_2CO_3 at 4 °C for 12 h. Aminodex−T-2 and BSA−T-2 were purified using the cold acetone precipitation procedure as described above.

Iodoacetate Activation of T-2 and Covalent Coupling to Carrier Antigens Using CDI. The hydroxyl group at position C-3 was converted to a carboxyl functionality by reacting T-2 toxin with IAc in anhydrous $CHCl_3$ to form methylcarboxylated T-2 (T-2-metCOOH). In brief, T-2 (2 mg) and iodoacetate (2 mg) were disolved in 200 μL of anhydrous $CHCl_3$ and allowed to react at 25 °C for 12 h. The mixture was then transferred to a separatory funnel (50 mL) containing 25 mL of water (pH 1−2) and shaken for 30 min to decompose the excess and unreacted IAc. The water fraction was discarded, and the procedure was repeated three times. The $CHCl_3$ fraction containing the T-2-metCOOH was dried with a vacuum dryer (Savant AS-160, Savant Instruments Inc., Farmingdale, NY) at 40 °C for 12 h. The conversion of T-2 to T-2-metCOOH was approximately 75% as determined by ^{1}H NMR (1.5H, singlet, σ 3.75 ppm, $-OCH_2COO^-$). The dried T-2-metCOOH was conjugated to protein using the CDI reaction described above.

Succinylation of T-2. The hydroxyl group at position C-3 was also converted to a carboxyl type functionality by reacting T-2 with succinic anhydride (T-2-hemisuccinate) according to the procedure of Chu et al. (1979). The T-2-hemisuccinate was then conjugated to BSA and OV using NHS-, MA- (Gendloff et al., 1986), or CDI-based reactions.

Citrinin (CTN). The C-8 and possibly the C-6 hydroxyl group of CTN was acetylated by acetic anhydride in the presence of pyridine. CTN (5 mg) was added to 1 mL of acetic anhydride and 0.5 mL of anhydrous pyridine. The mixture was allowed to react at 60 °C for 30 min. The product was spotted directly on a C_{18} reversed-phase TLC plate and developed with a (70:30) methanol/water solvent system. Two distinct blue fluorescent spots were observed, suggesting the presence of two compounds. These compounds were not isolated, nor were their structures determined. The hydroxyl groups were directly activated by CDI and conjugated to protein. In brief, 5 mg of CTN and 10 mg of CDI were dissolved in 0.2 mL of dimethyl sulfoxide and allowed to react at 25 °C for 2 min. The mixture was added to 20 mg of protein (BSA or OV) in 2 mL of 0.1 M Na_2CO_3 at 25 °C for 6 h. The conjugates were extensively dialyzed in water and then in pH 9.6 Na_2CO_3 buffer. The putative conjugate was spotted on a TLC plate and tested for conjugation as described for OA.

Direct Activation of Aflatoxin B_1 (AFB_1) and Covalent Coupling to Carrier Antigen. The C-3 carbonyl moiety in the cyclopentanone group of AFB_1 was converted to a carboxyl functionality by refluxing AFB_1 with *O*-carboxymethylhydroxylamine (AFB_1-oxime) in a mixture of pyridine/methanol/water (Chu et al.,1977). The carboxymethyloxime modified AFB_1 was then conjugated to protein using the CDI-based reaction described above.

Preparation of Rabbit Anti-T-2 and Anti-CTN Antisera. Dutch-belted rabbits, approximately 12 weeks of age, were immunized and boosted. The anti-T-2 and anti-CTN antisera were collected in accordance with standard animal care regulations. The immunogens were BSA−CTN and HSA−T-2-hemisuccinate conjugates that were prepared by the CDI and the NHS methods, respectively. The immogens (500 μg) were dissolved in 0.5 mL of 0.1 M saline and emulsified with an equal volume of Freund's complete adjuvant. The mixture was administered intradermally to 15 sites on the rabbits' backs. Boosters were given subsequently at 12 week intervals and consisted of 250 μg of T-2-hemisuccinate−HSA in 0.5 mL of saline emulsified with an equal volume of Freund's incomplete adjuvant. The boosters were given intradermally at 15 different sites on the backs of the rabbits. Blood was collected from the marginal ear vein following the final boost (approximately 131 days), and the serum was harvested and stored frozen in aliquots at −20 °C.

Indirect Competitive ELISA (cELISA) for OA, AFB_1, and T-2 Toxin. Unless otherwise stated, the conjugates were prepared by the CDI reaction. OV−OA, OV−OD, OV−CTN, OV−AFB_1-oxime, OV−AFB_1, BSA−T-2-hemisuccinate (prepared by the MA method), BSA−T-2-metCOOH, BSA−T-2 (synthesized using the IAc and CDI reactions), aminodex−OA, and aminodex−T-2 were added to the microtiter plate wells (200 μL) at a concentration of 12.5 μg mL^{-1} in 0.1 M phosphate-buffered saline at pH 7.2 (PBS) and allowed to incubate at 4 °C overnight. The plate wells were washed twice with PBS containing 0.01% Tween 20 (PBS-T) and then air-dried at 25 °C. Prior to analysis the microtiter plates were washed once with PBS-T. The following were added to the appropriate microtiter plate wells: 75 μL of pH 7.2 PBS-T, 10 μL of toxin standard diluted with methanol, and 65 μL of rabbit (polyclonal) or mouse (monoclonal) anti-OA, anti-CTN, anti-AFB_1, or anti-T-2 antibody diluted 1:1000, 1:1000, 1:1000, and 1:1000, and 1:500, 1:500, 1:500, and 1:500, respectively, in pH 7.0 PBS-T. Anti-OA IgY from the laying hen was diluted 1:1000. The wells were mixed thoroughly, and the plates were incubated for 1 h at 37 °C. The plates were emptied and the wells washed six times with PBS-T. Commercial alkaline phosphatase conjugated anti-rabbit IgG (1:5000), anti-mouse IgG (1:1000), or anti-chicken IgG (1:1000) was diluted with pH 7.0 PBS-T and added to all wells (150 μL). The wells were incubated for 1 h at 37 °C, emptied, and washed six times with PBS-T. Alkaline phosphatase substrate solution (1 mg mL^{-1} *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.8) was added to each well (150 μL) followed by incubation of the plate for 1 h at 37 °C or until the absorbency of the sample with no free toxin was greater than 1.5 OD. The plates were read directly on a microtiter plate reader at 405 nm (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada; Model 450).

RESULTS AND DISCUSSION

1,1′-Carbonyldiimidazole Activation of Hydroxyl- and Carboxyl-Containing Mycotoxins and Conjugation to Amine-Containing Compounds. The results from this study suggest the CDI-based reaction is able to produce linkages and orientations that are similar or identical to those obtained with other commonly used reagents for the coupling of T-2-hemisuccinate, OA, and AFB_1-oxime. Monoclonal and polyclonal antibodies raised using conventional chemistries to the mycotoxins T-2, OA, and AFB_1 reacted specifically and with good sensitivity to the new conjugates (Figure 3). The singular specificity of the monoclonal antibodies suggests the orientations of these conjugates are correct and not unique. This avoidance of uniqueness will facilitate the rapid development of new immunoassays using pre-existing monoclonal and polyclonal mycotoxin specific antibodies. In a model reaction, decarboxylated OA (Xiao et al., 1995) (DC-OA) was subjected to the CDI protein conjugation reaction. The putative OV−DC-OA failed to conjugate as indicated by the absence of a retained blue fluorescence in TLC analysis and a lack of response in the cELISA. Likewise, AFB_1 without modification with the *O*-carboxymethoxylamine hemihydrochloride failed to produce a fluorescent protein−toxin conjugate when tested using TLC. The absence of any response under these conditions demonstrates that the CDI reaction is only reactive with the carboxyl groups of these particular mycotoxins. Recently, rabbit polyclonal antibodies were raised against a CDI-prepared BSA−OA conjugate. They correctly recognized, in a cELISA, a coating antigen that was prepared using NHS (Clarke et al., 1994). The background absorbances were low and the antibody responses specific. These results suggest that the mycotoxin linkage group is properly oriented as this can profoundly influence the binding of the antibody and, consequently,

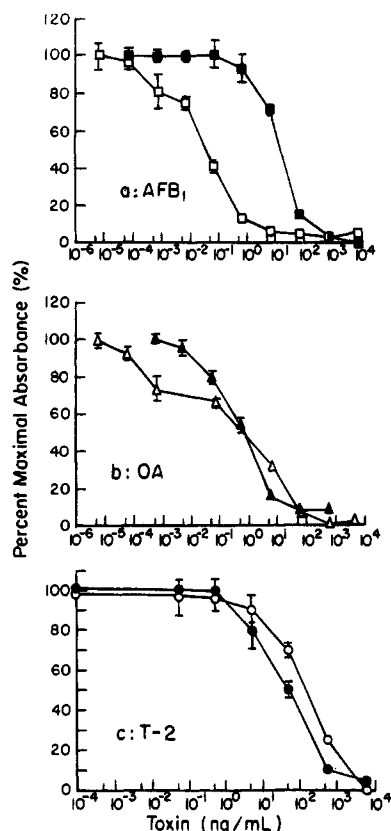

Figure 3. Reactivity and sensitivity of the coating conjugate antigens prepared by CDI chemistry as determined by preformed antibodies. (a) Coating antigen: OV−AFB_1-oxime. Antibodies were monoclonal anti AFB_1 (solid squares), and polyclonal anti-AFB_1 (open squares) from Sigma. (b) Coating antigen: OV−OA. Antibodies were monoclonal anti-OA (triangles) and rabbit polyclonal anti-OA (solid triangles; the immunogen was BSA−OA prepared by the CDI method). (c) Coating antigen: BSA−T-2. Antibodies were polyclonal anti-T-2 from Sigma (open circles) and rabbit polyclonal anti-T-2 (solid circles). The immunogen was HSA−HS-T-2 prepared according to the NHS method. See Materials and Methods for further details.

sensitivities and specificities in a cELISA (Candlish, 1991). The acylimidazole and imidazole carbamate intermediates formed by the CDI reactions are readily decomposed by water to gaseous CO_2, imidazole, and the original hapten (Hermanson et al., 1992). This rapid inactivation reduced the formation of new and unwanted immunodeterminants which are prevalent in the other reaction chemistries (Clarke et al., 1993; Gendloff et al., 1986).

Laying-hen antibodies that were specific for OA were developed with a BSA−OA conjugate prepared by the MA reaction. In the corresponding cELISA, high background absorbances were observed when the antigen was prepared with the same coupling chemistry (Table 1; Clarke et al., 1993). The same laying-hen antibody had substantially reduced background value when the chemistry for the synthesis of the coating conjugate was replaced with the NHS ester reaction. Substitution of MA chemistry with the CDI reaction for the preparation of the plate coating antigen also substantially reduced background values (Table 1). In an analogous situation, rabbit antibodies produced with an NHS prepared HSA−T-2-hemisuccinate conjugate failed to give an inhibitable response in a cELISA with BSA−T-2-hemisuccinate coating antigens that was prepared using the NHS reaction (Table 1). Conjugates of BSA−T-2-hemisuccinate that were prepared by either the MA or

Table 1. Effects of Coupling Chemistries on the Apparent Sensitivities and Background Absorbances of the ELISA[a]

coating antigen	antigen concentration[b] (ng/mL) IC_{20}	IC_{50}	IC_{80}	background[c] (%)
Chicken Anti-OA [Immunogen, BSA−OA (MA)]				
OV−OA (MA)	300	3000	UI[d]	75
OV−OA (NHS)	4	35	UI	25
OV−OA (CDI)	2	30	UI	20
aminodex−OA (CDI)	0.7	4	150	15
Rabbit Anti-T-2 [Immunogen, HSA−HS-T-2 (NHS)]				
BSA−HS-T-2 (NHS)	UI	UI	UI	100
BSA−HS-T-2 (MA)	UI	UI	UI	100
BSA−HS-T-2 (CDI)	UI	UI	UI	100
BSA−T-2 (CDI)	9	50	250	7
BSA−T-2-met-COOH (CDI)	7	40	300	4
aminodex-T-2 (CDI)	0.4	5	300	20
Rabbit Anti-CTN [Immunogen, BSA−CTN (CDI)]				
OV−CTN (CDI)	10	500	UI	43

[a] The procedure for the ELISA is described under Materials and Methods. In this study different conjugates were used to coat the plates with the bound toxin. Free OA was the competing antigen. [b] The sensitivity of the assay (IC_{20}, IC_{50}, and IC_{80}) is defined as the concentration of toxin that reduced the absorbency by 20, 50, or 80%, respectively, compared to that obtained in the absence of toxin. [c] The background absorbency (%) is the absorbency obtained at a high concentration of OA (5 μg/mL) divided by the absorbency obtained in the absence of toxin × 100. [d] UI, unattainable inhibition.

the CDI reaction also failed to lower backgrounds. However, the BSA−T-2 coating conjugates prepared directly from T-2 using CDI chemistry reduced the interference in the assay to less than 10% of maximal absorbance in a cELISA (Table 1). These results suggest that the T-2 antibody preparations had high cross-reactivities with the hemisuccinate bridging groups, and this can be reduced substantially by the use of CDI chemistries, chemistries that do not produce the undesirable bridging group.

Methylcarboxylation of the Hydroxyl Groups of Mycotoxins by IAc Followed by Their Conjugation to Amine-Containing Compounds. Rabbit anti-T-2 antisera developed with an HSA−T-2-hemisuccinate conjugate that was prepared using the NHS procedure was specifically recognized in a cELISA when BSA−T-2-metCOOH was used as a coataing antigen (Table 1). The conjugate was synthesized from the T-2-metCOOH derivative and BSA using the CDI reaction. These results demonstrate that IAc can be used to form a carboxylated derivative of T-2 toxin which can then be conjugated to an amine group in a protein using CDI.

Conjugation of Citrinin. Citrinin also appears to have been covalently coupled to BSA and OV using the CDI reaction. The conjugate, after extensive dialysis with water at a high pH, remained fluorescent when reacidified. It yielded two fluorescent bands when subjected to TLC. This may be attributed to the existence of citrinin as a diastereoisomeric mixture of two hydrates (Barber et al., 1987). The conjugate when spotted on a TLC plate also remained fluorescent after extensive denaturation with methanol and acetone, a procedure that would have removed all noncovalently bound citrinin. The BSA−CTN conjugates were immunogenic and produced a citrinin-specific antibody in rabbits capable of being used in a cELISA (Table 1). However, the CTN−protein conjugates appeared to yield high background values and to be unstable in aqueous buffer.

The conjugate should therefore be stored in a lyophilized state and used immediately when diluted with buffer for coating.

Toxin–Aminodex as a Coating Antigen in cELISA. Rabbit anti-T2 antibodies developed with an NHS-prepared HSA–T-2-hemisuccinate conjugate and laying-hen anti-OA developed with an MA-prepared BSA–OA conjugate reacted specifically and sensitively with aminodex–T-2 and aminodex–OA, respectively (Table 1). The backgrounds observed were substantially reduced in comparison to conventionally prepared NHS and MA protein–toxin coating antigens. Aminated dextran as a coating carrier may therefore be the carrier of choice for certain ELISAs due to its ability to yield low background values, its stability in organic solvents, and the ease with which it is modified with ethylamine.

Stability and Practical Advantages of CDI and IAc Chemistry. The ability of the CDI reaction to form a direct carbamate bond between the hydroxy group of an alcohol and the amine of a protein without the use of an intermediate conjugating reagent (Figure 2b) is beneficial as this reduces the number of reagents involved and the need to purify the carboxylated intermediate, it also produces a linkage that is considered to be stable (Hermanson et al., 1992), and it apparently minimizes bridge group interference. The main drawback of this reaction is its slow rate of reaction. The half-lives for the formation of CDI-activated hydroxyl groups at high pH are in hours; thus, reaction times tend to be slower than those seen using activated carboxyl groups. The half-life of a CDI-activated carboxyl group, in contrast (Figure 2a), is measured in minutes (Hermanson et al., 1992). Another alternative for the binding of a hapten with a hydroxy group to an amine is to first synthesize an intermediate containing a carboxyl group (reaction c or d, Figure 2) followed by a reaction between the carboxyl group and an amide using the CDI reaction (Figure 2a). Although the reaction using IAc (Figure 2d) is low in yield and slow in rate as compared to the succinylation reaction (Figure 2c), the IAc-induced carboxymethyl linkage is an ether-based bond, whereas the conventional hemisuccinate type linkages are ester-based. Ester linkages such as those found in ochratoxin A ethyl ester are readily hydrolyzed by extremes in pH and the action of esterases (Fuchs et al., 1972). Ether linkages, in contrast, are generally considered to be highly stable under a variety of environmental extremes (Hermanson et al., 1992). It could therefore be advantageous to use IAc to introduce an acetyl group into a hapten rather than the cyclic anhydride as it produces a linkage group that is not only different but stable.

Carboxyl groups, as indicated above, can be linked directly to an amine using the CDI reaction. The amide linkage between the carboxyl group of OA and a free amino group in a protein produced by this reaction has yielded an immune response in a rabbit (Clarke et al., 1994). The CDI-prepared antigen produced high titers and excellent antisera sensitivities and specificities in cELISA that were comparable with those of other antisera prepared using different coupling chemistries (Clarke at al., 1993). The ability of the CDI-prepared BSA–OA conjugate to induce a specific anti-OA response in rabbits demonstrates convincingly that the conjugate is stable and its orientation recognizable. The CDI reaction can therefore be used to directly link either a carboxyl (a rapid reaction) or a hydroxyl (a slow reaction) group to an amide.

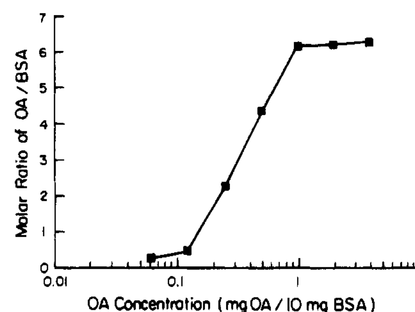

Figure 4. Relationship between the final amount of OA (activated form) that was added to a reaction mixture (abscissa) containing 10 mg of bovine serum albumin (BSA)/mL and the moles of OA that was covalently bound per mole of BSA. OA was activated directly with CDI and allowed to react with the lysine groups on the protein to form an amide bond. See Materials and Methods for further details.

Influence of a Cleanup Procedure on the Apparent Binding of OA to a Protein Conjugate. The epitope densities on the model BSA–OA conjugate could be easily controlled by varying the amount of OA in the CDI coupling reaction (Figure 4). Six moles of OA appears to be the maximal amount that can be bound per mole of BSA. A lowered epitope density on the coating conjugate should facilitate the development of a highly sensitive cELISA (van der Water and Haagsma, 1990; Li et al., 1994). This maximal epitope density is comparable to those of Worsaae (1978) but not those reported by Chu et al. (1982), as they reported as much as 31 mol of OA was bound per mole of BSA. The reasons for the large discrepancies in epitope densities are unknown but may be related to the ability of OA to bind serum albumins tightly and noncovalently (Chu, 1971). Therefore, an inadequate extraction of noncovalently bound OA could artificially increase the apparent amount of OA bound to the conjugate. In the current study complete removal of noncovalently bound OA was achieved by dialysis in a sodium carbonate buffer (pH 9.6) or by acetone protein precipitation. Cold acetone precipitation was also highly efficient and, in contrast to dialysis, was much more rapid. The cold acetone readily solubilized the free toxin while precipitating and concentrating the protein fraction. Two precipitations were required to remove all free OA as indicated by TLC. Acetone was therefore adopted as the method of choice with BSA and human serum albumin. Ovalbumin tended to be denatured by this procedure. As a consequence, OA conjugates prepared with OV were routinely dialyzed at a high pH. The ability to control the extent of conjugation and the quality of the conjugates should further facilitate the development of highly sensitive and reproducible mycotoxin immunoassays.

Conclusions. The highly desirable properties exhibited by the two coupling reactions (the CDI and IAc reactions) either alone (CDI) or in combination will facilitate the rapid development of sensitive and quantitative mycotoxin immunoassays. The chemistries are versatile, controllable, and readily performed using commercially available reagents. The chemistries yield stable and specific toxin–protein linkages that are properly orientated and therefore recognized by existing antibodies. These reagents minimize the formation of nonspecific and cross-reacting immunodeterminants on the plate coating antigens. Also, these procedures can potentially be used to conjugate other haptens with hydroxyl and carboxyl groups. The ability to aminate

dextran with ethylenediamine will broaden the range of chemistries currently used for conjugation of mycotoxins to a solid phase. The aminated dextran appears to be versatile and useful in the development of a cELISA. Finally, simplified procedures were utilized to remove haptens that were tightly but noncovalently bound from the hapten–protein complex. These procedures provided a basis for estimating the amount of toxin (OA) that was bound to the carrier protein.

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