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Short communication

Use of T-2 toxin-immobilized amine-activated beads as an efficient affinity purification matrix for the isolation of specific IgY

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ABSTRACT

An affinity purification method that isolates T-2 toxin-specific IgY utilizing a T-2-toxin-immobilized column was developed. The T-2 toxin was covalently coupled via a carbonyldiimidazole-activated hydroxyl functional group to amine-activated sepharose beads. The affinity-purified IgY was characterized by gel electrophoresis, fast protein liquid chromatography, enzyme-linked immunosorbant assay, surface plasmon resonance and mass spectrometry. A competitive inhibition ELISA (CI-ELISA) was performed using affinity-purified IgY with a T-2 toxin detection sensitivity of 30 ng/mL, which falls within the maximum permissible limit of 100 ng/mL. The cross reactivity of IgY towards deoxynivalenol, zearalenone, fumonisin B_1 and HT-2 was significantly reduced after affinity purification. A surface plasmon resonance (SPR)-based inhibition assay was also applied for quantitative determination of T-2 toxin in spiked wheat samples. The results obtained indicate the feasibility of utilizing this IgY-based assay for the detection of T-2 toxin in food samples.

1. Introduction

T-2 toxin (M.Wt. ~466.5 Da), a trichothecene A member, is produced by *Fusarium acuminatum*, *Fusarium poae* and *Fusarium sporotrichioides*. It is commonly found in cereal crops including wheat, maize, barley, oats and rye and processed grains e.g. in malt, beer and bread. It is highly resistant to temperatures employed during various food processing techniques and to UV light. This toxin is metabolized to HT-2 which leads to additive toxic effects when both forms are present in the food material simultaneously. T-2 toxin inhibits DNA, RNA and protein synthesis [1,2] and acts predominately on actively dividing tissues including bone marrow, lymph nodes, thymus and intestinal mucosa. The tolerable daily intake of T-2 toxin is 0.06 μg/kg body weight per day [3]. Epidemiological studies indicate that T-2 toxin affects the porcine and human innate immune systems [4] and also acts as apoptotic factor in human colon carcinoma cell lines [5]. In order to detect T-2 toxin, antibody-based assays with improved specificity and sensitivity over conventional methods are required.

The majority of T-2 toxin-specific antibodies developed to-date were obtained from leporine and murine species and only one report [6] described the successful usage of avian species in generating anti-T-2 toxin IgY antibodies. However, the antibody generated exhibited cross reactivity with other mycotoxins. Thus, there is a need to develop affinity purification methods for the isolation of toxin-specific antibodies, minimizing the cross reactivity of the polyclonal antibody preparation towards other similar toxins.

Herein, we described an affinity chromatographic method to isolate T-2 toxin-specific IgY using T-2 toxin-immobilized amine-activated sepharose beads. This IgY purification approach was proven to be efficient in isolating T-2 toxin-specific IgY. This IgY was used in the development of ELISA and surface plasmon resonance (SPR)-based assays for T-2 toxin.

2. Experimental

2.1. Chemicals

T-2 toxin, T-2 toxin stock standards (100 μg/mL), bovine serum albumin (BSA), key hole limpet hemocyanin (KLH), sinnapic acid, jeffamine, Freund's complete and incomplete adjuvant, carbonyldiimidazole,2-(N-morpholino)-ethanesulfonic acid (MES), sodium chloride (NaCl), glycine–HCl, Tris, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES),

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trifluoroacetic acid, acetonitrile, HRP-labelled anti-chicken secondary antibody and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich, Spruce Street, St. Louis, U.S.A. Amine-activated sepharose beads (CarboxyLink™ Coupling Gel) and the Eggcellent™ chicken IgY purification kit were obtained from Thermo Scientific. CM5 chips were purchased from GE Healthcare. 3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from KPL Inc., Gaithersburg, U.S.A.

2.2. T-2-toxin: conjugate synthesis and immobilization on amine-activated beads and CM5 chips

T-2 toxin (5 mg) was dissolved in dimethyl sulfoxide (100 μL), and 2.5 mg of carbonyldiimidazole was added and stirred for 15 min, at room temperature in the dark, to form T-2 carbamate. For T-2 conjugate synthesis, an ice-cold solution of either BSA or KLH (15 mg dissolved in 1 mL of 0.1 M Na_2CO_3 (pH 9.6) solution) was added to 7.5 mg T-2 carbamate. The reaction mixture was stirred at 4 °C overnight followed by stirring at room temperature for 1 h. The excess of T-2 carbamate was not quenched prior to buffer exchange. After buffer exchanging with 150 mM PBS (pH 7.4) in a Vivaspin column (M. Wt. 'cut-off': 10 kDa), the T-2-KLH conjugate (characterized by TNBS assay) was used as immunogen and T-2-BSA (characterized by MALDI-TOF) for screening purposes.

Immobilization of T-2 toxin on amine-activated beads was performed through pre-packing columns with amine-activated sepharose beads (3 mL), equilibrated with 10 mL of coupling buffer (0.1 M MES, 0.9% (w/v) NaCl, pH 4.7). Following equilibration, 3 mg of T-2-carbamate along with 0.5 mL of coupling buffer were added and mixed on a roller for 2 h at room temperature and then at 4 °C overnight.

Immobilization of T-2 toxin on a CM5 chip was performed manually on the bench by first equilibrating the chip with HEPES buffer (50 μl) at room temperature for 15 min. The chip was subjected to washing with 30 μL of de-ionized water after each individual step of the immobilization process. The chip was surface-activated by incubating at room temperature with 1:1 mixture of 0.4 M EDC/0.1 M NHS for 30 min, and washed. Following surface activation, 60 μL of 20% (v/v) of jeffamine, in 50 mM carbonate buffer (pH 8.5), was added to the chip and incubated for 1 h at room temperature. The un-reacted amine sites on the chip were blocked with 1 M ethanolamine (pH 8.5) for 30 min at room temperature. The T-2-carbamate (1 mg in 100 μL of DCM), diluted in 1:1 ratio with 10 mM of sodium bicarbonate solution (pH 8.0), was added to the chip and incubated for 24 h at room temperature.

2.3. Isolation and affinity purification of IgY from egg yolk

A leghorn chicken was immunized with 1 mL of 200 μg/mL T-2-KLH in Freund's adjuvant. The serum titre was determined after the third boost by indirect ELISA. Eggs were collected and IgY was isolated using an Eggcellent™ IgY purification kit following the standard protocol outlined in the manufacturer's instructions.

The T-2 toxin-immobilized amine-activated beads (3 mL) were packed in a column (length 7.4 cm and inner diameter 1.5 cm) and washed with 4 mL of wash buffer (1 M NaCl). The washing step was followed by equilibration of the column with 6 mL of 150 mM PBS (pH 7.4) containing 0.05% (w/v) sodium azide (for preservation purposes). The PBS used for equilibration was drained off and 5 mL of the crude egg yolk IgY solution was then added to the column and incubated at room temperature for 1 h. After incubation, the column was washed with 10 mL of sample buffer (1× PBS). The bound IgY was eluted using 8 mL of 100 mM glycine–HCl in water (pH 3.0). Each 1 mL eluted fraction of IgY collected was neutralized with 50 μL of 1 M Tris–HCl (pH 9.0).

2.4. Characterization of IgY (FPLC, SDS-PAGE, Western blotting and MALDI-TOF)

IgY purity was analyzed by gel filtration chromatography (Sephacryl S-200 HR column) on an AKTA explorer 100 (GE Healthcare, UK), monitoring absorbance at 280 nm. The mass analysis of IgY was performed by MALDI-TOF mass spectrometry (Bruker Analytical Systems). The data was acquired in the linear mode at 30 kV and analyzed using the Compass software suite (MALDI software). The IgY samples, from before and after affinity purification, were run on an NuPAGE 4–12% (w/v) Bis–Tris gel (Invitrogen, UK) SDS-PAGE gel under non-reducing conditions followed by Coommassie staining. The gel was stained for 1 h using Coommassie dye solution (consisting of Coommassie blue R-250 (2 g), methanol (450 mL), acetic acid (100 mL) and water (450 mL)) and destained overnight at room temperature using a mixture containing acetic acid, methanol, water in the ratio of 2:5:13. Western blotting of the SDS-PAGE gel was run using affinity-purified IgY under reducing condition and the gel was transferred onto the nitrocellulose membrane using a transblot (18 V for 16 min). Following transfer, the membrane was blocked for an hour with 4% (v/w) milk Marvel® and then probed with 1/5000 dilution of HRP-labelled anti-mouse secondary antibody for 1 h at room temperature. The membrane was subjected to washing (3× PBST) and then TMB substrate was added for visualization and image was captured with a G-Box hemi-16 gel documentation system (Syngene, UK).

2.5. Indirect ELISA using anti-T-2 toxin IgY

A 96-well Nunc microtitre plate was coated overnight at 4 °C with 100 μL/well of 5 μg/mL of T-2-BSA and blocked with 4% (w/v) milk Marvel® PBS for 1 h. A 100 μL volume of either the crude or affinity-purified IgY (1:100–1:512,000 dilution) was added to the individual wells of the microtitre plate and incubated for 1 h at 37 °C. Then 100 μL/well of 1:1000 dilution of HRP-labelled anti-chicken secondary antibody in 2% (w/v) milk Marvel® PBST was added and incubated at 37 °C for 1 h, followed by washing thrice with PBS Tween (PBST) and PBS. TMB substrate (100 μL/well) was added and incubated at room temperature for 20 min followed by stopping of the reaction with the addition of 50 μL/well of 1 M HCl. Absorbances were read at 450 nm on Tecan Saffire II Plate Reader.

2.6. Cross reactivity studies using anti-T-2 IgY (CI-ELISA)

Cross reactivities of both affinity-purified and unpurified IgY were determined by competitive ELISA. The ELISA procedure is similar to that described in Section 2.5, with one exception i.e. the use of 1/2500 dilution of antibody (affinity-purified and unpurified IgY) that was pre-incubated with known concentrations (0.007–1 μg/mL) of antigens (T-2, HT-2, deoxynivalenol, zearalenone and fumonisin B_1). The absorbance IgY in presence of antigen was divided by the absorbance of IgY alone (A/A_0). A semi-log plot was generated with A/A_0 values on the *Y*-axis versus log concentration of antigen on the *X*-axis.

2.7. Biacore inhibition assay

The CM5 chip, immobilized with T-2 toxin, was docked into the Biacore. This was followed by the injection of 1:1000 dilution of anti-T-2 IgY. Non-specific antibody (anti-AFB_1 Fab antibody) was used as a control to confirm that binding occurred only between the T-2 toxin and antibodies specific to the toxin. A 1/2500 dilution of the anti-T-2 IgY was incubated with equal volumes of known amounts of T-2 toxin (ranging from 0.007 to 1 μg/mL) and passed over the sensor chip at a flow rate of 15 μL/min for 90 s. The chip was regenerated with 15 μL of 40 mM NaOH and 30 mM HCl for 30 s

after each cycle at a flow rate of 30 μL/min. Response of the IgY in the presence of varying concentrations of T-2 toxin (R) was divided by the responses of IgY in the absence of T-2 toxin (R_0) and four parameter curve was fitted with R/R_0 value on the *Y*-axis versus log concentration of T-2 toxin on the *X*-axis.

2.8. Preparation of wheat sample and validation of assay

Wheat samples (10 g) were ground and extracted with 30 mL ethanol/water 85:15 (v/v) by constant stirring on a magnetic stirrer for 30 min. The extract was filtered through Whatman Grade 1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator and dissolved in 10 mL of 10 mM HBS buffer (pH 7.4), after which known amounts of T-2 toxins (0.007–1 μg/mL) were added and an SPR-based inhibition assay, as described in Section 2.7, performed. The LOD (limit of blank (LOB) + 3 S.D. of LOB) and LOQ (LOB + 10 S.D. of LOB) of the assay were determined.

3. Results and discussion

The T-2 toxin possessing a C3 hydroxyl functional group was activated with carbonyldiimidazole and used for coupling to the amine groups on KLH/BSA, the CM5 sensor chip and sepharose beads. T-2 immunogens may induce a slower immune response in both rabbits and mice due to immunosuppressive effects [7]. Hence, an alternative strategy of immunizing a chicken with a T-2-KLH conjugate, prepared by a modified CDI method [8] was adopted. The resulting immune response was found to have a serum titre in excess of 1/120,000. A total of 120 mg of IgY was isolated from each egg from an immunized chicken.

In order to isolate specific IgY, a T-2 toxin-affinity column, with 1.3 mg of T-2 toxin bound per mL of resin with a loading value of 22 μmol amine per millilitre of beads, was developed and used for affinity purification. The IgY recovery ranged from 10% to 20%, which falls within the reported range of 2–10% for antigen-specific antibodies in egg yolks [9]. Since pH plays a role in antibody elution [10], the effect of pH on the elution of the anti-T-2 IgY antibody was determined over a pH range of 2.0–11.0 and the optimized yield was obtained at pH 3.0 (data not shown). The column was used for five successive purifications with minimal loss in the column binding efficiency (the amounts of IgY eluted from five successive uses were 2, 1.91, 1.65, 1.32 and 1.09 mg). The T-2 toxin-immobilized amine-activated beads that were stored in PBS containing sodium azide were tested up to 1 month for their affinity purification retention capability and no obvious change was observed. The affinity-purified IgY was analyzed on FPLC,

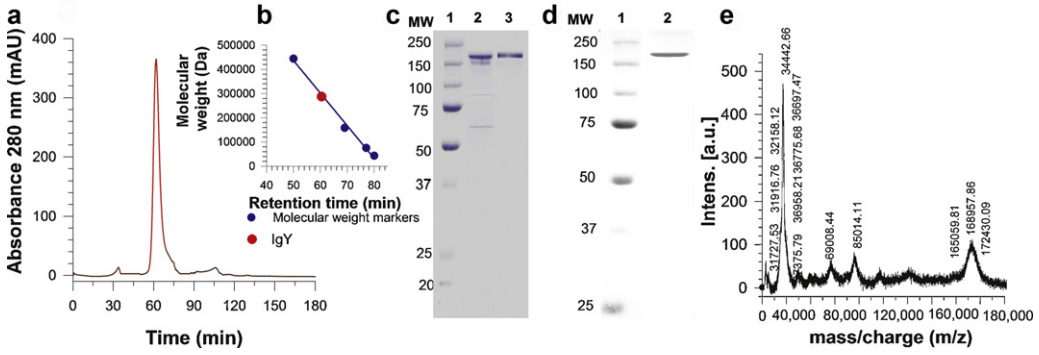

Fig. 1. FPLC, SDS-PAGE and MALDI-TOF analyses of affinity-purified IgY. Figure (a) indicates the FPLC peak of IgY alone. (b) A set of molecular weight markers Ovalbumin (43,000 Da), Conalbumin (75,000 Da), Aldolase (158,000 Da), Ferritin (440,000 Da) and IgY were passed through a Sephacryl S-200 HR column (with a 45 mL bed volume) and absorbance monitored at 280 nm. (c) SDS-PAGE analysis of IgY before and after affinity purification under non-reducing conditions. The representation of various lanes were: lane 1: BIO-RAD protein molecular weight ladder, lane 2: IgY before affinity purification, lane 3: IgY after affinity purification. (d) Western blotting of affinity purified IgY. The representation of various lanes were: lane 1: BIO-RAD protein molecular weight ladder, lane 2: affinity-purified IgY. (e) MALDI-TOF analysis of affinity-purified IgY was performed to determine the molecular mass of IgY. The mass of intact IgY range from 166 to 172 kDa. The MALDI-TOF data also indicates the presence of fragmented portions of IgY which correspond to the light (31–37 kDa) and heavy chains 68 kDa [12].

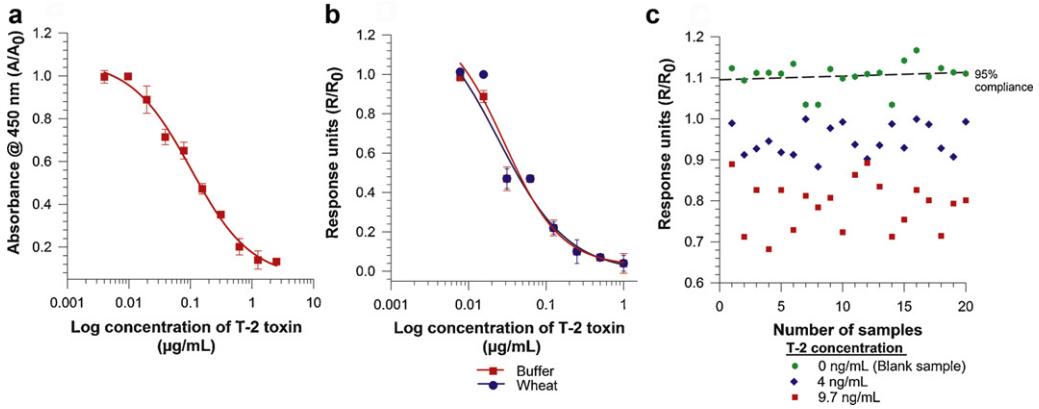

Fig. 2. ELISA and SPR-based competitive inhibition assays and LOD determination. (a) CI-ELISA was performed using 1/2500 dilution of affinity-purified IgY and the error bars indicate the S.D.s of three determinations made. The ratio of absorbance of antibody in presence of varying concentrations of T-2 toxin and antibody alone, which is indicated as A/A_0, was plotted on the *Y*-axis against the log concentration of T-2 toxin. (b) SPR-based inhibition assay was performed using both buffer and matrix. R/R_0 indicates the response units of IgY in the presence of varying concentrations of T-2 toxin divided by the response units of IgY alone. (c) Twenty replicates of blank standards, 4 ng/mL and 9.7 ng/mL of T-2 toxin were incubated with 1/2500 dilution of IgY and passed over the T-2 toxin-immobilized sensor chip. The dashed black line is the 95% compliance, which indicates the ability of antibody to differentiate between 19 of 20 positive samples with T-2 toxin from blank standards.

Table 1
Cross reactivity profile of IgY preparation before and after affinity purification.

Toxin	% cross reactivity	
	Before affinity purification	After affinity purification
T-2	100	100
HT-2	46	5
Deoxynivalenol	0.2	0.11
Zearalenone	0.15	0.12
Fumonisin B_1	0.32	0.21

The IC_{50} value of competitive ELISA performed using T-2 toxin was taken as 100% and the percentage cross reactivity was calculated based on the IC_{50} value attained using the other antigens.

SDS-PAGE, western blotting and MALDI-TOF (Fig. 1). The efficiency of the affinity purification technique was evaluated through indirect ELISA using 1 mg/mL of IgY (before and after affinity purification) and through cross reactivity studies using T-2, HT-2, deoxynivalenol, zearalenone and fumonisin B_1 (Table 1). An increased titre, after affinity purification, with reduction in the cross reactivity of IgY towards HT-2 toxin from 46% to 5% was recorded, which indicates the elimination of immunoglobulins that are not specific to T-2 toxin from the IgY pool. The decreased cross reactivity of IgY after subjecting to affinity purification on T-2-immobilized ligand indicates that this purification technique can readily be applied for the isolation of mono-specific antibodies in order to increase the specificity of immunoassay without changing the intrinsic properties of the antibody itself.

The SPR-based inhibition assay (Fig. 2b) was performed to confirm the results of the competitive inhibition ELISA (Fig. 2a). The effect of matrix on the SPR-based assay performance was evaluated using spiked extracts of wheat sample at different T-2 levels (0.007–1 μg/ml). The IC_{50} for determination of T-2 toxin in buffer and matrix were 30 ± 0.1 ng/mL and 32 ± 0.1 ng/mL (Mean ± S.D., $n = 3$), respectively, with LOD's of 8 ± 0.15 (buffer) and 11 ± 0.23 (matrix) ng/mL (Mean ± S.D., $n = 3$) and LOQ's of 12 ± 0.21 ng/mL (buffer) and 24 ± 0.19 ng/mL (matrix) using affinity-purified anti-T-2 IgY (Fig. 2c). Precision of the assay was determined by intra- ($n = 3$) and inter-day ($n = 5$) assays which resulted in coefficient of variance (CVs) less than 12%. The results indicate that the affinity-isolated IgY can be successfully used for the detection of T-2 toxin in wheat samples with detection levels within the permissible limits of 100 μg/kg [11].

4. Conclusions

Affinity purification on T-2 toxin-immobilized beads increased the specificity of anti-T-2 IgY through minimizing its cross reactivity to HT-2 toxin. CI-ELISA and SPR-based assay were successfully developed. In addition, the effect of matrix on assay sensitivity was determined indicating that use of this IgY in conjunction with the SPR-assay format can be readily used for the specific detection of T-2 toxin in wheat sample extracts.

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