Development of enzyme sorbent immunoassay for rubratoxin B

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Summary

A polyclonal antibody for Rubratoxin B (RB) was raised in rabbit and the competitive direct ELISA method to detect RB was set. Optimum concentration of antibody and enzyme conjugate were 1:1000 and 1:1500, respectively. Fifty % inhibition for RB at these conditions were about 5 ng/ml. This ELISA system can be used up to 50 % methanol or 20 % acetonitrile for solvent of sample solution. Aflatoxins (B1, B2, G1 and G2), cyclopiazonic acid, kojic acid, patulin, ochratoxin A, sterigmatocystin and zearalenone did not inhibit this reaction. However, unknown interferences from various agricultural commodities were observed and further more study is needed to apply this method to real samples.

Key words: rubratoxin B, ELISA, polyclonal

Introduction

Rubratoxin B (Fig. 1) is a secondary metabolite produced by Penicillium rubrum, P. purpurogenum and P. crateriforme by infected feed and food stuffs. After
consuming such materials, the RB enters the organs of human and animals and then causes serious disorders. Numerous pathological symptoms by the consumption of mold-infected feed are well recognized in swine, cattle and poultry. The toxicity of RB in human cells under in vitro studies is well documented. The first case of human rubratoxicosis by drinking toxic rhubarb wine led to liver failure was reported by Singler et al.

Simple diagnostic tools need to be developed for the detection and quantification of RB. ELISA-based assay for many mycotoxins have been developed and are validated, however, there is no report suggesting the availability of ELISA for RB. Since, immunoassays are inexpensive and fulfill the requirements of sensitivity, simplicity and reliability hence, a need was felt (i) to develop a simple and cost effective method for the detection and quantification of RB in feed and food matrices to ensure food safety and (ii) to make available a handy tool for use in molecular toxicological studies.

**Materials and Methods**

**Materials** All chemicals, phosphate buffered saline (PBS, pH 7.4), washing buffer: PBS + 0.1 % Tween 80, coating buffer: carbamate buffer 50 mM, pH 9.2, substrate solution: 3,3',5,5'-tetramethyl benzidine dihydrochloride (TMB: Sigma T-8665, MO, USA), and stop solution: 0.5 M H₂SO₄ were either commercially available one or mixed with commercially available ones. Rubratoxin B was purchased from Sigma Co. Ltd. RB-BSA conjugate and RB-HRP conjugate were synthesized according to the method described by Davis and Stone. 96 well flat bottom microplate (No. 353915, Becton Dickinson & Co, NJ, USA) was used throughout study. Color development of the reaction was measured by microplate reader (model 450 and model 550, Japan Bio-Rad, Tokyo). Plate was washed by DIA WASHER II (DIA-IATRON Co. Ltd, Tokyo).

**Production of antibody** A white Japanese rabbit was immunized using RB-BSA conjugate for 9 weeks. Anti-RB activity was checked through 9 weeks and serum was collected after 9 weeks from starting of immunization.

**Plate preparation and assay** Anti-RB serum was diluted by carbamate buffer and 100 μl portion of the solution was poured into each well of the plate and kept at 4°C overnight. Plate was washed by washing buffer three times. Two percent gelatin or 5 % skimmed milk PBS solution was used for blocking. Blocking was done at 30°C for 2 hr. After blocking, plate was washed by washing buffer three times and used for assay. 50 μl of sample and 50 μl of RB-HRP solution was mixed in the well and kept at 30°C for one hour, then washed by washing buffer for four times. Fifty μl of TMB solution was poured into the well and kept for 10 to 30 min at ambient temperature. Reaction was stopped by adding 50 μl of stop solution, and the developed color was measured at 450 nm.
Results and Discussion

Anti-rubratoxin B serum production  A white Japanese rabbit was immunized five times at 0, 2, 4, 6 and 8 weeks and serum was collected one week after immunization. Anti-RB activity was measured by sandwich ELISA method. As shown in Fig. 2, anti-RB activity went up at 7 and 9 weeks, therefore we collected whole serum at 9 weeks and used this serum for this study.

![Graph showing the raising of rubratoxin B antibody by rabbit.](image)

**Fig. 2. Raising rubratoxin B antibody by rabbit.**

- ○: 3 weeks, ■: 5 weeks, △: 7 weeks, ■: 9 weeks

Solvent tolerance of the antibody  Rubratoxin B standard was dissolved in 50, 20 and 10 % of methanol-PBS and 50, 20 and 10 % acetonitrile-PBS solutions. At 50 % acetonitrile solution, no response curve was obtained. As shown in Fig. 3, compared with PBS, about 30 % of response and 50 % of responses were obtained by 20 % acetonitrile-PBS and 50 % methanol-PBS solution, respectively. However, from 10 % acetonitrile solution and 20 and 10 % methanol-PBS solutions, more than 80 % of responses were obtained. These results show this antibody has relatively good tolerance for methanol and acetonitrile, and up to 20 and 10 % of methanol and acetonitrile, respectively, can be used as sample solvent, respectively.
Optimization of ELISA condition  Antibody concentration were tested at the range from 1:250 to 1:2000, and 1:1000 was found to be optimum for coating wells. Skimmed milk at the concentration of 5 % in PBS and gelatin at 2 % in PBS showed stable results for blocking among protein solutions tested. Concentration of enzyme conjugate was tested from 1:100 to 1:2000 range. Optimum color development was observed at 1:1500 dilution. At these condition, 50 % inhibition (IC₅₀) value was ca. 5 ng/ml and 0.5 ng/ml of RB in sample solution was quantitatively determined.

Interferences by other mycotoxins and agricultural commodities  To determine the cross reaction of the antibody with other mycotoxins, aflatoxins B₁, B₂, G₁ and G₂, sterigmatocystin, ochratoxin A, patulin, cyclopiazonic acid, kojic acid and zearalenone were tested at the concentration of 50 and 200 μg/mL. None of these mycotoxins interfered the RB determination in this system. Effects of extracts from various agricultural commodities were also tested. 80 % acetonitrile and 80 % methanol were used as extraction solvent and gelatin and skimmed milk were used as blocking proteins. As shown in Table 1, some extracts showed strong inhibition of the reaction, thus further studies are required before to apply this ELISA method to agricultural products.
Table 1. Percent inhibition of anti-rubratoxin B antibody in agricultural commodities.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methanol</th>
<th>Acetonitrile</th>
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<tbody>
<tr>
<td></td>
<td>Skimmed milk</td>
<td>Gelatin</td>
</tr>
<tr>
<td>Coconut</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td>Soybean</td>
<td>81</td>
<td>77</td>
</tr>
<tr>
<td>Maize</td>
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<td>77</td>
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<td>Rice</td>
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<td>49</td>
</tr>
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<td>Milo</td>
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<td>72</td>
</tr>
<tr>
<td>Peanut</td>
<td>38</td>
<td>48</td>
</tr>
</tbody>
</table>

* Sample extracted by 80% methanol and 80% acetonitrile and diluted 4 times and 8 times in PBS, respectively.
** Blocked by 5% skimmed milk or 2% gelatin in PBS.

References