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Safety Assessment of Bacterial Choline Oxidase Protein Introduced in Transgenic Crops for Tolerance against Abiotic Stress

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Genetically modified crops have resistance to abiotic stress by introduction of choline oxidase protein. In the present study, the safety of choline oxidase protein derived from *Arthrobacter globiformis* was assessed for toxicity and allergenicity. The protein was stable at 90 °C for 1 h. Toxicity studies of choline oxidase in mice showed no significant difference ($p > 0.05$) from control in terms of growth, body weight, food consumption, and blood biochemical indices. Histology of gut tissue of mice fed protein showed normal gastric mucosal lining and villi in jejunum and ileum sections. Specific IgE in serum and IL-4 release in splenic culture supernatant were low in choline oxidase treated mice, comparable to control. Intravenous challenge with choline oxidase did not induce any adverse reaction, unlike ovalbumin group mice. Histology of lung tissues from choline oxidase sensitized mice showed normal airways, whereas ovalbumin-sensitized mice showed inflammed airways with eosinophilic infiltration and bronchoconstriction. ELISA carried out with food allergic patients' sera revealed no significant IgE affinity with choline oxidase. Also, choline oxidase did not show any symptoms of toxicity and allergenicity in mice.

KEYWORDS: Choline oxidase; toxicity; allergenicity; GM plants; *Arthrobacter globiformis*

INTRODUCTION

Genetically modified (GM) crops hold out the promise of global food security. The potential benefits of genetic modification include crops with disease and pest resistance, improved quality food, resistance to environmental stresses, and specialized roles in industrial/pharmaceutical realms (*1, 2*). Despite the immense potential benefits, concerns have been raised about the safety of transgenic crops. Hence, there is a need to evaluate the safety of these products before human or animal consumption. Several recommendations were provided for safety evaluation by international organizations including the Food and Agriculture Organization/World Health Organization, *Codex Alimentarius*, and the Organization for Economic Co-operation and Development (*3, 4*).

It is important to establish that any protein introduced in a GM crop does not represent a previously described toxin or allergen. The safety of *Bacillus thuringiensis* (Bt) toxins is assured by digestibility and lack of intrinsic activity in animal system (*5, 6*). Earlier, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) protein introduced in crops for herbicide glyphosate tolerance showed no characteristics associated with food toxins or allergens (*7*). However, the Ber e1 gene (Brazil nut protein) expressed in GM soybean and transgenic pea derived α-amylase inhibitor was allergenic (*8, 9*). These studies indicate that foreign protein introduction should be carefully tested for toxicity and allergenicity.

Environmental changes have prompted researchers to introduce abiotic stress genes to certain crop plants (*10*). Choline oxidase from *Arthrobacter globiformis* has been expressed in many transgenic crops such as rice, tobacco, tomato, mustard, and *Arabidopsis* (*11*−*15*). Earlier studies with GM mustard expressing choline oxidase showed immunoreactivity comparable with that of native mustard (*16*). As the gene for choline oxidase is taken from bacteria and has not been used in foods previously, there is a need to assess the safety of this bacterial protein. In the present study, choline oxidase protein from *A. globiformis* was investigated for toxicity and allergenicity in mice.

MATERIALS AND METHODS

Test Material and Reagents. Choline oxidase protein (molecular mass ∼ 60 kDa) from *A. globiformis* and other reagents were procured from Sigma Chemical Co. (St. Louis, MO).

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Heat Stability of Purified Protein. The effect of temperature on the choline oxidase was examined following the method of Herouet et al. (*17*). Briefly, choline oxidase was dissolved in 20 mM Tris-HCl and 5 mM ethylenediaminetetraacetic acid buffer at a concentration of 0.025 mg/mL. The protein was heated at 75 or 90 °C for 15, 30, 45, and 60 min in a temperature-controlled heating block. The assay was terminated by ice cooling of sample with the addition of 14 μL of distilled water and 14 μL of Laemmli buffer (pH 6.8). The heat-treated protein was analyzed by SDS-PAGE and Western blot for comparison with untreated protein.

SDS-PAGE and Immunoblot. Pretreated choline oxidase was loaded onto a 12% acrylamide gel, and a constant voltage of 110 v was applied. To assess the immunoreactivity, resolved protein was electrophoretically transferred onto nitrocellulose membrane, blocked with defatted milk, and incubated with antibodies (1:1000 v/v) raised against purified choline oxidase (*18*). After a washing with phosphate buffer saline (PBS), it was further incubated with anti-mouse IgG−horseradish peroxidase (1:1000 v/v) and developed using *o*-phenyldiaminobenzoate substrate.

Acute Toxicity Studies. *Animal Feeding.* Six-week-old female Balb/c mice (weight = 18−20 g) were randomly segregated into two groups of 10 mice each. Mice were kept on regular diet, allowed free access to water, and maintained on a 12:12 h light/dark cycle in an environmentally controlled chamber. The temperature and humidity were maintained at 23 ± 2 °C and 55 ± 10%, respectively. Group 1 mice were gavage fed daily for 42 days with PBS, and group 2 mice were fed choline oxidase. The dosage of choline oxidase was 10 mg/kg of body weight. The animals were observed daily for activity, fur color, food consumption, and excretion. Body weight was measured every week for each mouse.

The Animal Ethics Committee of the Institute of Genomics and Integrative Biology approved the study protocol.

Blood Biochemistry. Blood samples from the choline oxidase group and control mice were collected on day 42 for hematological analysis. Serum biochemical indices were determined by standard clinical methods. Total plasma protein, urea, creatinine, serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT) levels were determined for liver and kidney functioning using commercial kits (Span Diagnostics Pvt. Ltd., India). Total and high-density lipid (HDL) cholesterol levels were measured for cardiac functioning (Monozyme Pvt. Ltd., India).

Histological Studies. The gut epithelial tissues were collected from the sacrificed mice and fixed immediately in 10% neutral-buffered formalin (v/v). After overnight fixation, tissues were processed for histology. The gut tissue was embedded in paraffin, sliced to 3 μm sections, and stained with hematoxylin-eosin (HE) for analysis of antigen-induced inflammation. Sections were scanned under a light microscope, and images of these fields were captured by a light microscope with an in-line camera (Nikon TE 2000-S) and assembled into multipanel figures using Photoshop software (version 7.0, Adobe version).

Allergenicity Studies. *Allergenicity Testing in Mice.* Six-week-old female Balb/c mice (weight = 18−21g) were randomly segregated into three groups of seven mice each. Group 1 mice were given PBS daily by ip route. Groups 2 and 3 were given ovalbumin (OVA; 95% purified, chicken albumin) and choline oxidase, respectively (100 μg of protein in 100 μL of PBS) by ip route once a week for 6 weeks. Blood samples were collected from the tail vein to measure serum IgE, IgG1, and IgG2a antibodies on days 15, 30, and 43. Mice were challenged iv on day 45 with 3, 6, and 12 mg of OVA or choline oxidase protein in PBS, and symptoms were scored. The mice were sacrificed on day 45 for splenic cell culture and lung tissue histology.

Ovalbumin and Choline Oxidase Specific Antibodies. Specific antibodies for OVA and choline oxidase were measured in serum by ELISA (*16*). Briefly, the microtiter plates (Maxisorp, Denmark) were coated with 250 ng of OVA or choline oxidase protein(s) in carbonate buffer (pH 9.6). The plates were incubated with mouse sera for IgE (1:10), IgG1, and IgG2a (1:500) estimation. After washing, rat anti-mouse IgE (1:1000, Bethyl Laboratories), rat anti-mouse IgG1 (1:10000, BD Pharmingen), and rat anti-mouse IgG2a (1:1000, BD Pharmingen) in PBS were added and developed.

Splenic Cell Culture. Spleens were removed aseptically on day 45, from three mice in each group, and cells were isolated and cultured to study cytokines profile. Single-cell suspensions were depleted of red blood cells by lysis buffer and centrifuged at 1200 rpm. The cells were resuspended in RPMI-1640 medium with 10% (v/v) heat-inactivated fetal bovine serum, 10 units/mL penicillin, and 100 μg/mL streptomycin. Five million cells per milliliter was seeded in a 96-well flat-bottom culture plates. For stimulation of the cells, OVA or choline oxidase (100 μg of protein/mL) was added to cultures, separately. Phytohemeagglutinin (5 μg/mL) was used as a positive control, and no stimulant was added to negative control. Cells were cultured for 72 h at 37 °C in a humidified incubator with a 5% CO_2 environment. Supernatant was harvested after 72 h and stored at −70 °C for cytokine estimation by ELISA. Cells were incubated for 2 h with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 100 μg/mL], lysed in acidic isopropanol, and read at 450 nm.

Cytokine Estimation. IL-4 and IFN-γ levels in culture supernatants were determined by sandwich ELISA following the manufacturer's instructions (BD Pharmingen, San Diego, CA). Capture antibody (1:250 v/v) for each cytokine was coated separately on microtiter plates in carbonate buffer, pH 9.6, and incubated overnight at 4 °C. After washing, wells were blocked with PBS containing 10% fetal bovine serum (FBS) at 37 °C for 1 h. After washing, 100 μL of standards (seven serial dilutions), diluted sample (1:2 v/v), and controls were added to wells and incubated at 37 °C for 3 h. The plates were washed again and incubated with biotinylated detector antibody labeled with avidin HRP at 37 °C for 1 h. The substrate was added after seven washings, and plates were incubated in the dark at 37 °C for 30 min. The reaction was stopped with 2 N H_2SO_4 and read at 450 nm. The cytokine concentrations were deduced from the standards. The detection limits for IL-4 and IFN-γ were 7.8 and 31.3 pg/mL, respectively.

Lung Histology. Lungs were removed from sacrificed mice and fixed immediately in 10% neutral-buffered formalin (v/v) for histology. The sections (3 μm) were cut, stained with hematoxylin and eosin, and visualized under a light microscope (Nikon TE 2000-S) for antigen-induced peribronchial and perivascular inflammation.

ELISA with Purified Choline Oxidase Using Patients' Sera. IgE affinity of the purified protein was tested by ELISA with sera of food allergic patients having symptoms of allergic asthma, rhinitis, or both collected at the outpatient department of the V.P. Chest Institute, Delhi. It is a referral chest hospital and receives patients from different parts of the country for diagnosis and treatment of allergy and asthma. For the present study, the diagnosis of asthma was ascertained in cases following the guidelines of the American Thoracic Society (*19*). The patients having any two of the symptoms, namely, sneezing, rhinorrhea, nasal blockage, postnasal drip, etc., over the past 2 years were diagnosed as rhinitis (*20*). The patients aged 15−45 years were skin prick tested with various pollens, fungi, insects, and food allergen extracts. Of 45 patients' sera tested, 30 showed raised (≥3 times of control) specific IgE to one or more food allergens. The remaining 15 patients had elevated specific IgE against pollens ($n = 5$), fungi ($n = 5$), and insect allergens ($n = 5$) in addition to at least one food allergen. Sera from five nonallergic subjects were used as control. ELISA values ≥3 times controls were taken as cutoff for positive results. The study protocol was approved by the Human Ethics Committee of the Institute.

Statistical Analysis. The Student *t* test (unpaired) was used for statistical analysis of the data. $p < 0.05$ was considered to be statistically significant.

RESULTS

Heat Stability. Choline oxidase resolved at 60 kDa on 12% SDS-PAGE. Heat-treated protein at 75 or 90 °C for 60 min showed no degradation and reacted with choline oxidase antibodies on immunoblot (**Figure 1**).

Acute Toxicity Studies. *Blood and Clinical Parameters.* The mice fed choline oxidase protein for 42 days appeared to be normal in terms of growth, development, activity, food consumption, excretion, etc. The average body weight gained per

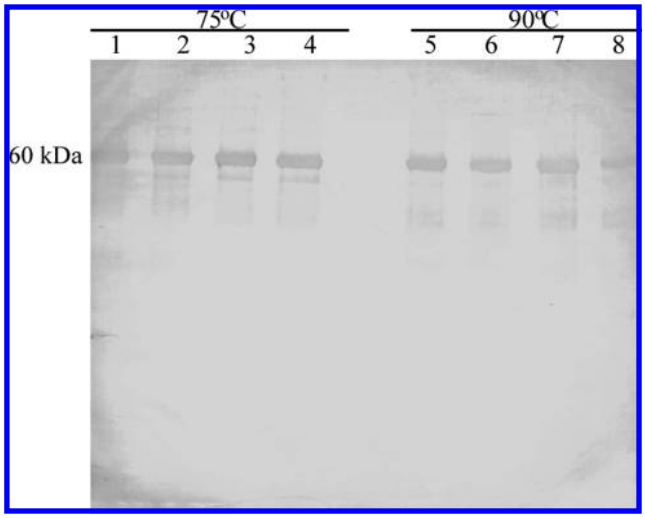

Figure 1. Heat stability of choline oxidase protein. Choline oxidase was heated at 75 or 90 °C for 0, 15, 30, and 60 min. SDS-PAGE separated protein was analyzed by Western blot with antibodies against choline oxidase. Laned 1−4, choline oxidase heated at 75 °C for 0, 15, 30, and 60 min; lanes 5−8, choline oxidase heated at 90 °C for 0, 15, 30, and 60 min.

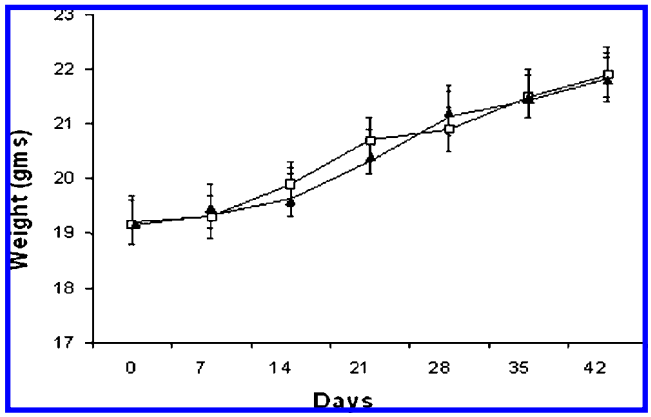

Figure 2. Body weight of Balb/c mice: weight records of mice fed choline oxidase (□) and phosphate-buffered saline (▲) during the 6 week study. Weight was recorded every week. Data are presented as means ± SE.

week showed no significant difference ($p > 0.05$) between mice fed choline oxidase and those fed PBS (**Figure 2**).

Mean values of hematological and biochemical parameters obtained from mice fed choline oxidase protein or PBS are presented in **Table 1**. Total plasma protein and urea levels were similar for choline oxidase and control group mice. The serum creatinine level in choline oxidase group was not significantly different ($p > 0.05$) from control, indicating normal kidney function. SGPT and SGOT levels were comparable in both groups, indicating normal liver function. Total and HDL cholesterol levels in mice fed choline oxidase were slightly less than control group; however, no statistically significant difference ($p > 0.05$) was found in these two indices. The liver, heart, and kidney functions of choline oxidase and control mice were comparable; therefore, these tissues were not used for histological examination.

Gut Histology. Analysis of hematoxylin and eosin-stained gastric mucosa tissues in mice fed choline oxidase revealed normal structure with no distortion in gut lining. The gastric mucosal layer showed smooth parietal cells, indicating no inflammation and/or infiltrations of inflammatory cells into

Table 1. Blood Biochemistry Indices of Mice Fed Choline Oxidase Protein for 6 Weeks

parameter	control	choline oxidase
	Hematological Values[a]	
lymphocyte (%)	57.3	60.7
eosinophil (%)	0.9	1.0
monocyte (%)	2.6	2.5
neutrophil (%)	37.2	35.8
	Serum Chemistry[a]	
plasma protein (g/dL)	7.9 ± 2	8.01 ± 1.6
urea (mg/100 mL)	54.2 ± 3.6	60.1 ± 7.9
nitrogen (mg/100 mL)	25.2 ± 3.6	27.9 ± 7.9
creatinine (mg/dL)	0.18 ± 0.1	0.2 ± 0.1
SGPT (IU/L)	52.0 ± 2.5	54.0 ± 5.9
SGOT (IU/L)	91.6 ± 10.5	88.6 ± 12.6
total cholesterol (mg/dL)	266 ± 12	291 ± 10
HDL cholesterol (mg/dL)	20.6 ± 06	22.5 ± 10

[a] Data presented as mean ± SEM.

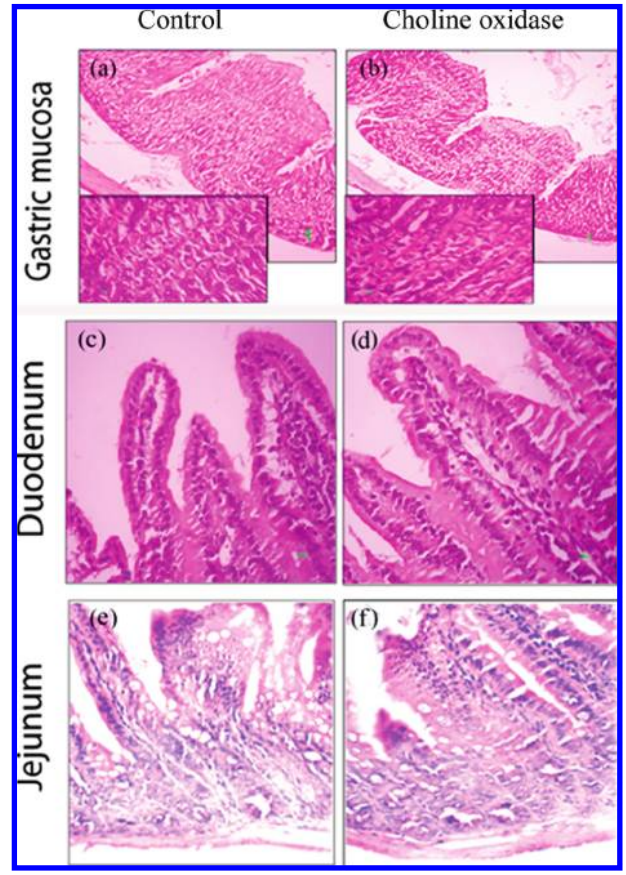

Figure 3. Gut histology of mice: hematoxylin and eosin staining of gut mucosa, (**a**) PBS control and (**b**) mice fed choline oxidase; sections showing duodenum villi;, (**c**) PBS control and (**d**) mice fed choline oxidase; sections showing jejunum villi, (**e**) PBS control and (**f**) mice fed choline oxidase.

surrounding tissues (**Figure 3a**, **b**). Duodenal villi of mice fed choline oxidase showed normal surface area with numerous absorptive enterocytes cells and no sign of ulcer, bleeding, or disruption-like symptoms and were comparable to that of control mice (**Figure 3c**,**d**). Jejunum villi revealed normal enterocytes and mucous-secreting cells. No abnormal structure/symptoms were observed in mice fed choline oxidase, similar to controls (**Figure 3e**,**f**).

Allergenicity Studies. *Immune Response to Choline Oxidase Protein.* Sensitization with choline oxidase by ip route in mice showed low IgE response (OD 0.016) similar to that in control

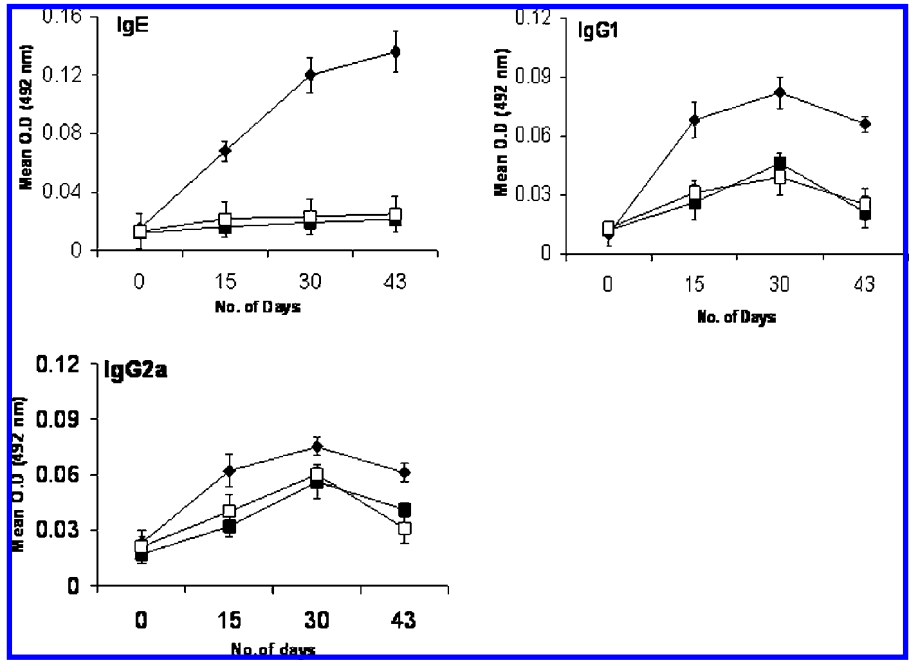

Figure 4. Specific antibody response in Balb/c mice: serum antibody response following ip administration of PBS control (□), ovalbumin (◆), and choline oxidase (■) groups; (**a**) specific IgE (**b**) specific IgG1; (**c**) specific IgG2a.

Table 2. Symptom Score[a] in Presensitized Mice Challenged with Different Doses of Proteins

mouse no./ protein dose (mg)	PBS (score)	ovalbumin (score)	choline oxidase (score)
1/12	0	1, 2, 3, 4, 5	1
2/12	1	1, 2, 3, 4, 5	0
3/6	0	1, 2, 3	0
4/6	0	1, 2, 3, 4, 5	0
5/3	0	1, 2, 3, 4	1
6/3	0	1, 2, 3, 4, 5	0

[a] Symptom score: 0, no symptoms; 1, scratching, rubbing around the nose and head; 2, puffiness around the eyes and mouth, pilar erecti, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth; 4, symptoms as for 3 with loss of consciousness, tremor, and/or convulsion; and 5, mortality (death).

(OD 0.017) ($p > 0.05$). In contrast, ip administration of OVA produced higher IgE titer on day 15 (OD 0.068) ($p < 0.05$), which increased further by day 30 (OD 0.12) (**Figure 4a**).

Choline oxidase induced low IgG1 response (OD 0.026) in mice, similar to control (OD 0.027) (**Figure 4b**). The protein induced little IgG2a level on day 15 (OD 0.032), comparable to control (OD 0.040) ($p > 0.05$), which increased slightly on day 30 (OD 0.056). However, on day 45, there was a reduction in IgG2a response in both choline oxidase and control group mice. Ovalbumin initiated more IgG1 (OD 0.082) and IgG2a (OD 0.075) response than choline oxidase ($p < 0.05$) (**Figure 4c**).

Antigen Challenge of Sensitized Mice. Intraperitoneally sensitized mice were challenged iv with different doses of antigen. Choline oxidase did not induce any symptoms of anaphylaxis in mice challenged with 3−12 mg of protein. The challenge with OVA produced symptoms of anaphylaxis including tremor, convulsion, irritation, etc. Mice challenged with higher doses of OVA demonstrated severe symptoms leading to death due to anaphylaxis (**Table 2**).

Histology of Lungs. OVA-sensitized and -challenged mice showed perivascular and peribronchial inflammatory cell infiltrate with loss of normal lung structure (**Figure 5b**). In contrast, choline oxidase-sensitized/challenged mice showed normal lung structure with defined bronchial epithelial lining and alveoli (**Figure 5c**). The cellular infiltration of inflammatory cells in the lung tissue of the choline oxidase group was also similar to that of control mice (**Figure 5a**).

Cytokine Levels in Spleen Cell Culture. IL-4 and IFN-γ were determined by ELISA in spleen cell culture supernatant of sensitized mice. OVA-sensitized mice had significantly increased levels of Th2 cytokine, that is, IL-4 (29 ± 5 pg/mL) compared with control ($p < 0.05$) (7 ± 2 pg/mL). The levels of IL-4 were low in cell culture supernatant in choline oxidase-sensitized mice (8 pg/mL), similar to control (**Figure 6a**). The IFN-γ levels did not differ between OVA- and choline oxidase-sensitized mice (**Figure 6b**).

IgE Reactivity of Choline Oxidase Protein with Patients' Sera. Analysis of specific IgE affinity of choline oxidase with food allergic patients' sera is presented in **Figure 7**. The specific IgE values against choline oxidase with atopic patients' sera ($n = 45$) ranged from 0.3 to 0.7 IU/mL, whereas the levels for controls were 0.3−0.5 (mean = 0.4 IU/mL). None of the patients showed ≥3-fold higher values for choline oxidase compared with controls.

DISCUSSION

GM foods require careful safety assessment before use for human consumption. The safety should be addressed in complementary ways including the characterization and assessment of the expressed protein and evaluation of the transgenic plant product (*17*). Earlier, transgenic *Brassica juncea* expressing choline oxidase protein showed immunoreactivity similar to that of its native counterpart using animal model and in vitro methods. Bioinformatics analysis of choline oxidase showed no significant homology in allergen databases (structural database of allergenic proteins, Food Allergy Research and Resource Program, and Swiss-Prot) and degraded completely in simulated gastric fluid (*16*). In the present study, purified choline oxidase protein was investigated further for toxicity and allergenicity in a mouse model and screened with hypersensitive patients' sera.

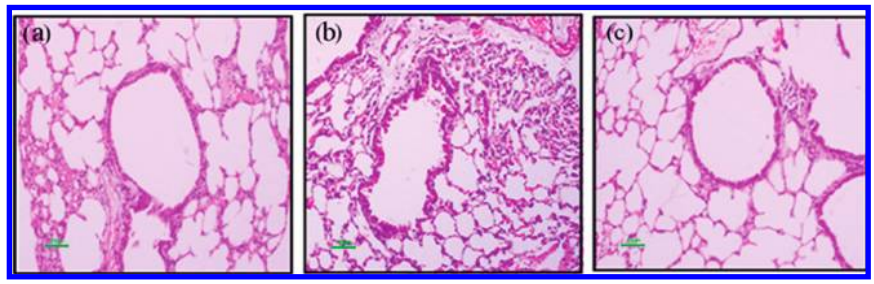

Figure 5. Lung histology of sensitized/challenged mice: hematoxylin and eosin staining of lungs, (**a**) PBS control, (**b**) ovalbumin, and (**c**) choline oxidase administered mice.

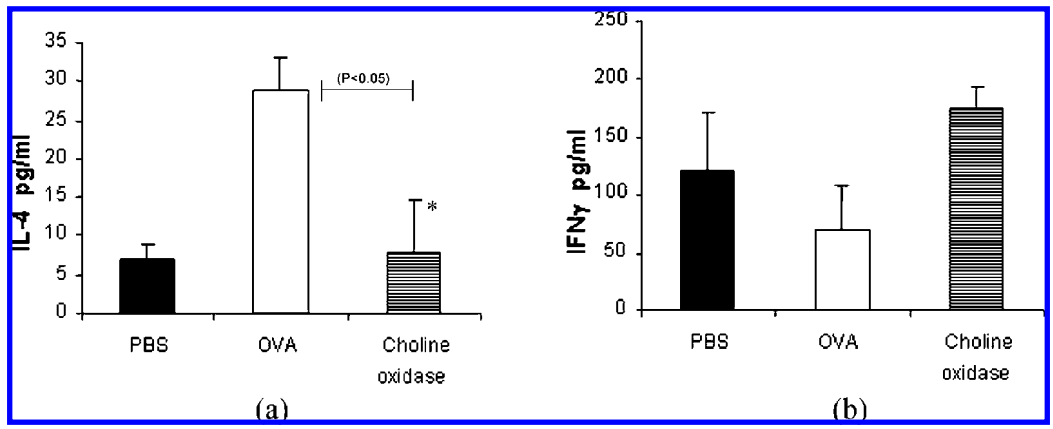

Figure 6. Cytokine levels in spleen cells culture supernatant: cytokine levels in spleen cell culture supernatant following ip administration of ovalbumin, choline oxidase, and PBS control, (**a**) IL-4 and (**b**) IFN-γ. Data are presented as means $\pm$ SE. An asterisk ($*$) indicates a statistically significant value ($p < 0.05$).

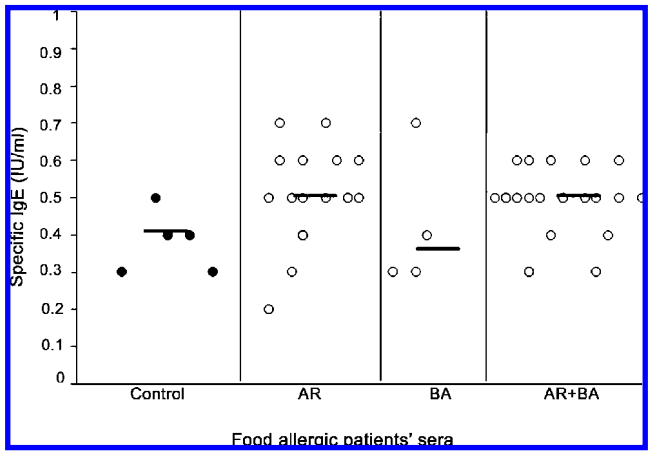

Figure 7. IgE affinity of choline oxidase with food allergic patients' sera: clinical characteristics of patients and specific IgE values against choline oxidase in patients' sera by ELISA. Specific IgE values ≥ 3 times of control were taken as cutoff for the positive results. AR, allergic rhinitis; BA, bronchial asthma; AR+BA, both; (●) control; (○) patients' sera; (—) median value.

The significant factors contributing to the toxicity and allergenicity of food protein are stability to heat and digestion (*21*). A heat-stable protein has a greater probability of damaging intestinal mucosa and inducing systemic allergenicity on absorption (*22*). Cry9c protein expressed in GM soybean was heat stable and nondigestible, which led to the removal of the crop from the market (*23*). Choline oxidase showed degradation on treatment with simulated gastric fluid (*16*). However, in the present study choline oxidase protein showed stability and retained immunoreactivity after 1 h of heat treatment at 75 or 90 °C.

Safety assessment of purified protein and whole food is essential as both may show different levels of penetration in the system. Oral administration of green fluorescent protein (GFP) was nontoxic for growing rats, but there was a slight decline in weight gain and food efficiency ratio in rats fed GM canola expressing GFP protein (*24*). The mice fed GM potato expressing *Galanthus nivalis* lectin showed proliferation of gastric mucosa as revealed by jejunum and ileum villi (*25*). Furthermore, mice fed GM potato expressing the *cry 1* gene showed mild changes in the ileac compartment as compared to the control group (*26*). In the present study, Balb/c mice fed choline oxidase showed no significant difference ($p > 0.01$) in body weight and food consumption compared to the PBS control group. Blood biochemical indices in the choline oxidase group indicated normal functioning of metabolic organs and were comparable to those of the control mice. Also, the choline oxidase fed mice demonstrated normal appearance of gut mucosal tissues and intestinal villi. It seems that consumption of choline oxidase protein has no detrimental effect on the growth and metabolic function of mice.

Food allergy is primarily mediated by allergen-specific IgE antibodies that induce Th-2 response. The stability of proteins during thermal processing makes them more likely to behave as true food allergens as is the case with most of the lipid transfer proteins (*27*). In the present study, choline oxidase showed stability toward heat, which makes it more susceptible to absorption in gut. However, Balb/c mice administered choline oxidase showed low IgE response as compared to ovalbumin, which showed high IgE response. IL-4 levels were also low in choline oxidase administered/stimulated spleen cell culture supernatant. ELISA with food allergic patients' sera demonstrated low IgE affinity with choline oxidase similar to control. In an earlier study, purified EPSPS protein showed no significant IgE response by skin test and ELISA in atopic subjects (*28*).

Plant-derived foods are complex, and consuming constituents with high biological activity, even in minor quantity, can have major effects on the gut and body's metabolism (*6*). Safety studies with whole food as well as purified protein are required to identify the adverse effect (*17*). Previously, transgenic proteins expressed in prokaryotic systems were used for toxicity or allergenicity assessment (*7, 17*), but it would be interesting to study transgenic proteins expressed in genetically modified plants (eukaryotic). Transgenic protein(s) synthesized in plants will be glycosylated and may have different properties.

In conclusion, the bacterial choline oxidase protein appears to be safe in terms of toxicity and allergenicity. Further study with the purified protein form actually produced in GM *B. juncea* will be worthwhile to ensure the complete safety of the GM product.

ABBREVIATIONS USED

Bt, *Bacillus thuringiensis*; GM, genetically modified; HE, hematoxylin−eosin; OVA, ovalbumin; PBS, phosphate-buffered saline.

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