

Micro-assay Method for Evaluating the Allergenicity of the Major Soybean Allergen, *Glym* Bd 30K, with Mouse Antiserum and RBL-2H3 Cells

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A micro-assay method for evaluating the allergenicity of soybean allergen was developed by using the mouse antiserum against *Glym* Bd 30K, a major soybean allergen, and RBL-2H3, a rat mucosal mast cell line. The antiserum against *Glym* Bd 30K was prepared by subcutaneously immunizing BALB/c mice with the allergen. The behavior by affinity-chromatography and the properties against heat treatment show that the reaginic activity of the antiserum resided in the IgE antibody specific for *Glym* Bd 30K. The developed assay method is shown to be useful for simulating IgE mediated type-I allergy and to be highly sensitive for detecting the allergen.

Key words: allergy; reagin; soybean; *Glym* Bd 30K; RBL-2H3

A number of patients with atopic dermatitis suffer from food allergies, soybean being considered to be one of the major allergenic foods eliciting adverse reactions.¹⁾ Soybean allergy may become a serious problem due to the increasing utilization of soybean in food processing as a substitute for animal proteins. Its allergenicity is known to reside mainly in the protein fractions.²⁾ Herian *et al.*³⁾ have reported the occurrence of the three different groups of allergenic proteins, with molecular masses of 50–60 kDa, 20 kDa, and 14 kDa, in soybean. Ogawa *et al.* have reported that about 65% of the sera from Japanese soybean-sensitive patients with atopic dermatitis possessed IgE antibodies specific for a protein with a molecular weight of about 30,000, and named it *Glym* Bd 30K.⁴⁾ *Glym* Bd 30K has been demonstrated to be identical with the soybean 34 kDa oil body-associated protein,⁵⁾ which has been reported by Herman and his colleagues as a soybean storage protein (P34) in vacuoles.^{6–8)}

In developing hypoallergenic products, a convenient assay method that uses a suitable substitute for patients to evaluate their allergenicity is desirable. RBL-2H3, a rat mucosal cell line, expresses a high affinity receptor for IgE (FcεR I), and RBL-2H3 cells can release mediators for inflammation in response to the cross-linking of FcεR I molecules.⁹⁾ We have previously reported that the allergenicity of the Kunitz-type soybean trypsin inhibitor (KSTI) could be assessed not only by a conventional *in vivo* passive cutaneous anaphylaxis (PCA) reaction, but also by an *in vitro* assay using the set of RBL-2H3 and anti-KSTI serum.¹⁰⁾ KSTI is a potent experimental allergen to BALB/c mice, and its allergenicity toward humans has also been reported^{11,12)}; however, it might only be a minor allergen for soybean-allergic patients with atopic dermatitis. Accordingly, measuring the allergenicity of *Glym* Bd 30K is considered to be more valuable than that of KSTI in the course of developing hypoallergenic soybean products. Thus, we wanted to establish a convenient assay method for this purpose.

In this paper, we describe the preparation of the antiserum having reaginic activity in response to *Glym* Bd 30K, and an *in vitro* assay method that employs the set of the antiserum and RBL-2H3 cells which can simulate the allergic reaction of patients for detecting the allergenicity attributable to *Glym* Bd 30K.

Materials and Methods

Materials. The materials used in the present study were obtained from the sources indicated: RBL-2H3 cells from the Cancer Cell Repository (CCR), Tohoku University (Sendai, Japan); BALB/c mice and Wistar rats from SLC Japan (Shizuoka, Japan); MAbTrap™ GII and HiTrap™ NHS-activated from Pharmacia Biotech (Uppsala, Sweden); ECL Western blotting detection reagents from Amersham (Buckinghamshire, U.K.); polyclonal anti-mouse IgE from The Binding Site (Birmingham, U.K.); peroxidase-labeled monoclonal anti-mouse IgE from SeroTec (Oxford, U.K.); peroxidase-labeled polyclonal anti mouse IgG from Cappel (Durham, NC, U.S.A.); peroxidase-labeled anti-mouse IgG1, IgG2a, IgG2b, and IgG3 from Caltag (CA, U.S.A.); minimum essential medium, penicillin-streptomycin, trypsin plus ethylenediamine-tetraacetic acid, and L-glutamate from GIBCO BRL (NY, U.S.A.); fetal calf serum from Irvine Scientific (St. Ana, CA, U.S.A.); and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide from Seikagaku Corporation (Tokyo, Japan).

Preparation of *Glym* Bd 30K. *Glym* Bd 30K was prepared from the oil body pad of soybean as previously described.⁵⁾

Immunization of the mice. BALB/c mice were fed with soybean free diets for several generations to prevent any tolerance which might be evoked by soybean intake. Adult mice were subcutaneously injected four times at two-week intervals with 0.3 ml of the immunogen solution containing 0.2 mg/ml of *Glym* Bd 30K and 15 mg/ml of aluminum hydroxide. A week after the last immunization, the whole serum of the mice were pooled and stored below –20°C until needed for use. The pooled serum was used in the subsequent experiments.

Immunoblotting. Proteins were loaded and subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).¹³⁾ The proteins that developed on the gel were electrophoretically transferred on to a nitrocellulose membrane, which was then cut into three parts. One part was stained with Coomassie brilliant blue, while the other two parts were immunoblotted against the antiserum. Fixed IgE on the membrane was detected with peroxidase-conjugated goat anti-mouse IgE and ECL reagents, and fixed IgG with peroxidase-conjugated sheep anti-mouse IgG and 4-chloro-1-naphthol.

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Abbreviations: KSTI, Kunitz-type soybean trypsin inhibitor; PCA, passive cutaneous anaphylaxis; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

Adsorbing of immunoglobulins. Two affinity gel columns were prepared for adsorbing the immunoglobulins in the antiserum: (1) for IgE, an anti-mouse IgE gel column made by fixing anti-mouse IgE on HiTrap™ NHS-activated according to the manufacturer's instructions. (2) for IgG, Mab Trap™ GII was used. The antiserum was applied to each column, and the columns were washed with a solution having the same volume as that of the applied antiserum. The flow-through fraction and washings were combined.

Enzyme-linked immunosorbent assay (ELISA). One hundred microliters of mouse antiserum was incubated for 1 h at 37°C in microplate wells coated with *Glym* Bd 30K to detect the antigen-specific IgG subclasses, or in wells coated with the anti-mouse IgE monoclonal antibody to detect total IgE contained in the serum. After washing with phosphate-buffered saline containing 0.05% Tween 20 (PBS/Tween), the wells were incubated at 37°C for 1 h with peroxidase-conjugated sheep anti-mouse IgG1, IgG2a, IgG2b, IgG3, or IgE diluted by PBS/Tween containing 10% sheep serum. The immunocomplexes in the wells were incubated for 20 min at room temperature with *o*-phenylenediamine (0.4 mg/ml) and 0.01% H₂O₂ as the substrate in a 0.1 M citrate/0.1 M phosphate buffer (pH 5.0), and the absorbance of the reaction mixtures in the wells at 492 nm was determined with a micro-plate Model 450 reader (Bio-Rad, CA, U.S.A.).

Cell culture. RBL-2H3 cells were maintained in MEM supplemented with 10% heat-inactivated FCS, 4 mM L-glutamate, 100 U/ml of penicillin, and 100 µg/ml of streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. The cells were harvested by treating with 0.25% trypsin and 0.1 mM EDTA, and recovered by centrifugation (250 × *g* for 5 min at room temperature).

Assay for β -N-acetylhexosaminidase release. The activity of β -N-acetylhexosaminidase released from the stimulated RBL-2H3 cells was determined as previously described.¹⁰⁾ In brief, RBL-2H3 cells inoculated into a 96-well microplate (1 × 10⁵/well) were incubated with a medium containing the anti- or non-immunized (control) serum for 1 h at 37°C. The cells were successively incubated with the antigen solution for 1 h at 37°C, and the β -N-acetylhexosaminidase activity released in the supernatant was assayed. Aliquots of the cells were dissolved in a solution containing 0.2% Nonidet-P40 to measure the total β -N-acetylhexosaminidase activity of the cells.

Passive cutaneous anaphylaxis reaction. Fifty microliters of the diluted serum was injected intracutaneously into the back skin of anesthetized Wistar rats. Two days later, 1 ml of a saline solution including 0.1% of the antigen and 0.5% of Evans blue was intravenously injected. Blue-spot weals appearing on the back skin were measured to evaluate the allergic reaction.

Results

To obtain an antiserum suitable for studying the IgE-mediated allergic reaction evoked by *Glym* Bd 30K, serum

from BALB/c mice immunized with *Glym* Bd 30K was collected and analyzed by immunoblotting against *Glym* Bd 30K. Figures 1b and 1c reveal that the serum contained not only IgE, but also IgG specific for *Glym* Bd 30K. We then separated the IgE and IgG from each other, because reaginic activity attributable to mouse IgG1 has been reported.¹⁴⁾ Immunoglobulin E in the antiserum was adsorbed in an anti-mouse-IgE antibody-fixed column (anti-IgE column), and IgG was adsorbed in a protein G-fixed column (Protein G column). Both of the flow-through fractions were investigated by ELISA for their contents of IgG-subclasses specific for *Glym* Bd 30K or total IgE in the antiserum. Figure 2 shows that the antiserum contained all subclasses of IgG specific for *Glym* Bd 30K and that they were significantly reduced in the flow-through fraction from the protein G column, whereas they almost completely remained in the flow-through fraction from the anti-IgE column. On the other hand, total IgE was significantly reduced in the flow-through fraction from the anti-IgE column, whereas it was almost completely retained in the flow-through fraction of protein G column. Accordingly, we call the flow-through fraction from the anti-IgE column as IgE-removed antiserum, and that from the protein G column as IgG-removed antiserum, in this paper. The reaginic activity in the intact antiserum, IgE-removed antiserum and IgG-removed antiserum was next investigated. As shown in Fig. 3, the IgE-removed antiserum had markedly lost its reaginic activity in both assays with RBL-2H3 and the conventional PCA reaction, whereas the IgG-removed antiserum retained most of its original activity. In the case of the IgE-removed antiserum, slight reaginic activity was observed in both experiments due to a small amount of remaining IgE. The difference between the IgE-removed antiserum and IgG-removed antiserum was evident when both reaginic activities were compared in a PCA reaction using the 1/10 diluted antiserum.

It has been demonstrated that the reaginic activity of IgE was reduced by heat treatment.¹⁵⁾ Thus, we investigated the effect of heat treatment of the antiserum at 56°C to confirm whether the reaginic activity of the antiserum might be attributable to IgE. In Fig. 4, we compare the reaginic activity of the heated antiserum with that of the intact antiserum in both the RBL-2H3 assay and PCA reaction. This figure shows that the reaginic activity of the antiserum was strikingly reduced by a 30-min incubation at 56°C and was completely inactivated by a 4-h incubation. The reaginic activity shown in the RBL-2H3 assay were consistent with that in the PCA reaction. We conclude from Figs. 3 and 4 that IgG specific for *Glym* Bd 30K in the antiserum had no or only a negligible influence on the reaginic activity, and that most of the reaginic activity existing in the antiserum is attributable to IgE.

Finally, the RBL-2H3 cell assay method with the antiserum was investigated. When RBL-2H3 cells were incubated with an increasing concentration of anti-*Glym* Bd 30K serum, the activity of released β -N-acetylhexosaminidase increased in response to the increasing concentration (Fig. 5a). In contrast when RBL-2H3 cells were stimulated by an increasing concentration of *Glym* Bd 30K, the activity of released β -N-acetylhexosaminidase also increased steadily up to the concentration of 10 ng/ml, an inflection point, but the degree of increase tailed off thereafter (Fig.

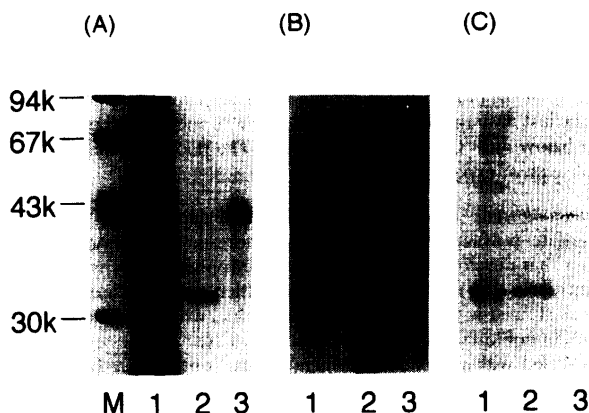


Fig. 1. Immunoblotting with the Anti-*Glym* Bd 30K Serum.

A, stained with Coomassie Brilliant Blue; B, stained with peroxidase-conjugated anti-mouse IgE; C, stained with peroxidase-conjugated anti-mouse IgG. In each panel: lane 1, whole soyprotein extract; lane 2, purified *Glym* Bd 30K; lane 3, ovalbumin (negative control). M, molecular marker proteins.

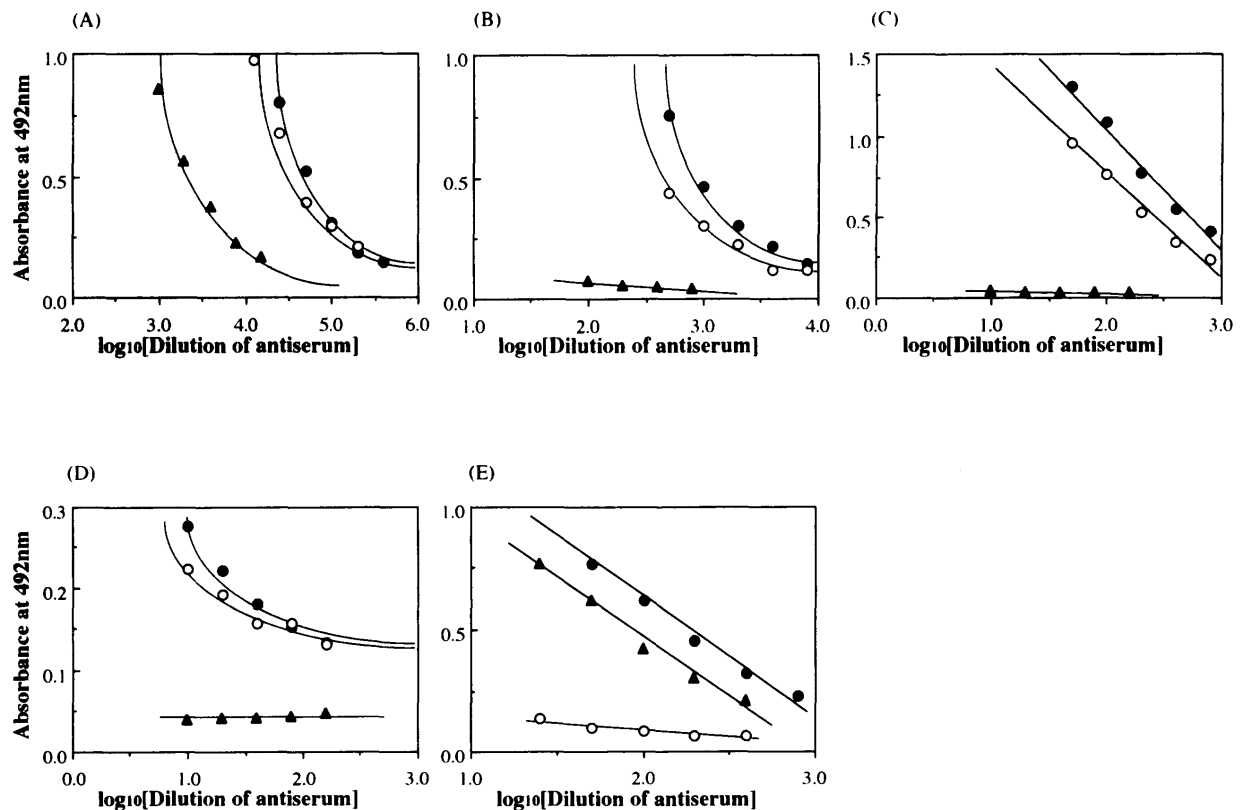


Fig. 2. Detection of *Gly m* Bd 30K-specific IgG Subclasses and Total IgE Contained in the Anti-*Gly m* Bd 30K Serum by ELISA.

(A) IgG1, (B) IgG2a, (C) IgG2b, (D) IgG3, (E) IgE.

For detecting the *Gly m* Bd 30K-specific IgG subclasses, a 96-well microplate coated with *Gly m* Bd 30K was used, and for detection total IgE, a 96-well microplate coated with the anti-mouse IgE monoclonal antibody was used. Solid circles show the intact anti-*Gly m* Bd 30K serum, open circles, the flow-through fraction from the anti-IgE column, and solid triangles the flow-through fraction from the protein G column. Data are the mean of triplicate determinations.

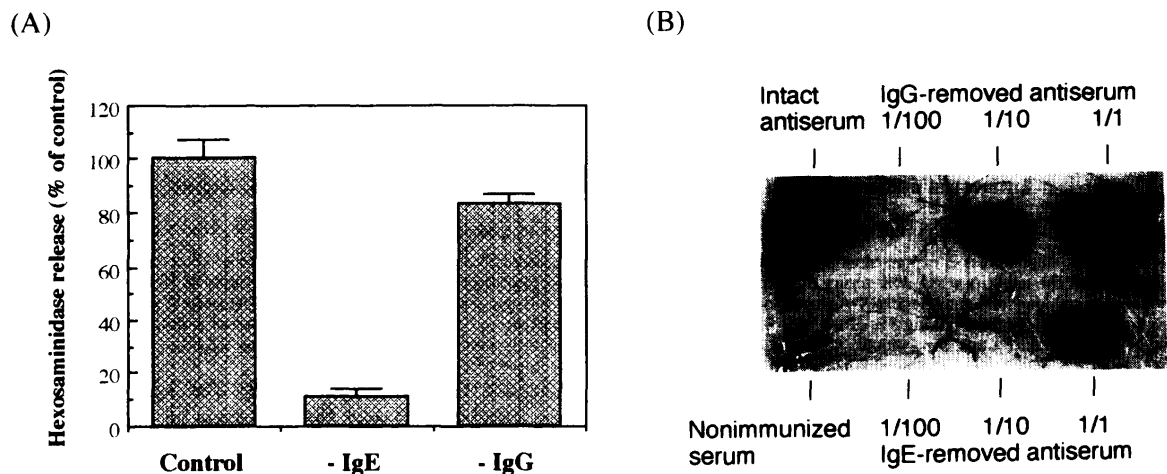


Fig. 3. Effect of Removing the Immunoglobulin Classes on Reaginic Activity in the RBL-2H3 Assay and PCA Reaction.

A: RBL-2H3 cells were pre-incubated with the intact (control) or the immunoglobulin class-removed anti-*Gly m* Bd 30K serum. The β -N-acetylhexosaminidase release from cells stimulated by 10 μ g/ml of *Gly m* Bd 30K is expressed as a percentage of the control release. Bars are means $\pm$ SD ($n=3$). B: A rat was pre-injected with the intact or immunoglobulin class-removed anti-*Gly m* Bd 30K serum at a given titer prior to the allergen injection. Intact antiserum was used after dilution by the same volume of phosphate-buffered saline, because the immunoglobulin class-removed antiserum was diluted by the washing solution.

5b). The spontaneous release was almost constant with no correlation to the concentration of *Gly m* Bd 30K, and the specific (antiserum-mediated) release exceeded the spontaneous release in a range of more than 300 pg of *Gly m* Bd 30K per milliliter.

The foregoing results show that the anti-*Gly m* Bd 30K serum, which had reaginic activity mediated by IgE specific for the antigen, was produced and that the antiserum could

be applied to the *in vitro* assay method with RBL-2H3 for detecting the allergenicity attributable to *Gly m* Bd 30K.

Discussion

We have here reported the production of IgE recognizing *Gly m* Bd 30K by immunizing BALB/c mice with the purified allergen, indicating the immunogenicity of *Gly m* Bd 30K. On the other hand, when the extracted whole soy-

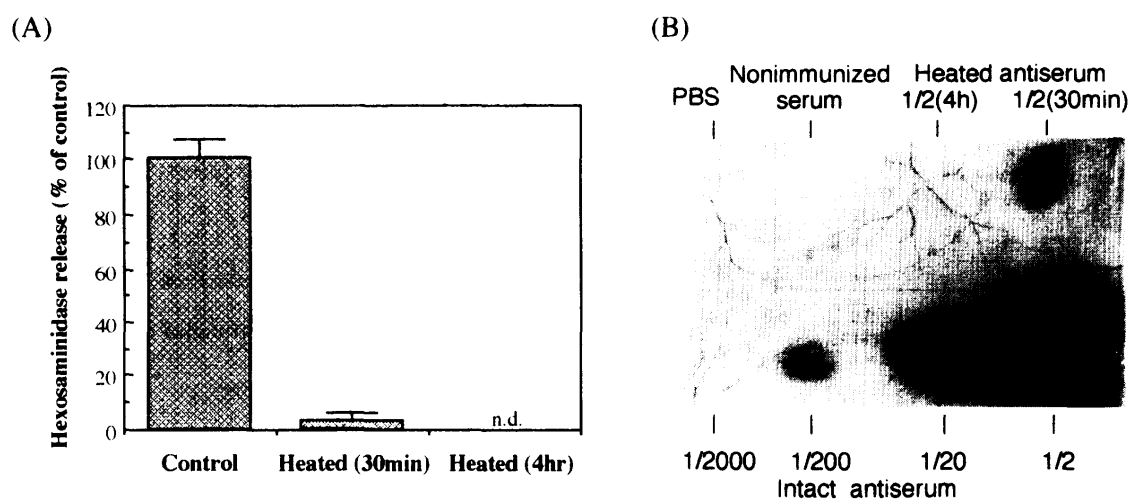


Fig. 4. Effect of Heat Treating the Antiserum on Reaginic Activity in the RBL-2H3 Assay and PCA Reaction.

A: RBL-2H3 cells were pre-incubated with the intact (control) or heat-treated anti-*Glym* Bd 30K serum. The β -*N*-acetylhexosaminidase released from cells stimulated by 10 μ g/ml of *Glym* Bd 30K is expressed as a percentage of the control release. Bars are means $\pm$ SD ($n=3$). n.d. means "not detected." B: A rat was pre-injected with the intact or heated anti-*Glym* Bd 30K serum at a given titer prior to the allergen injection. Periods of heating are shown in parentheses.

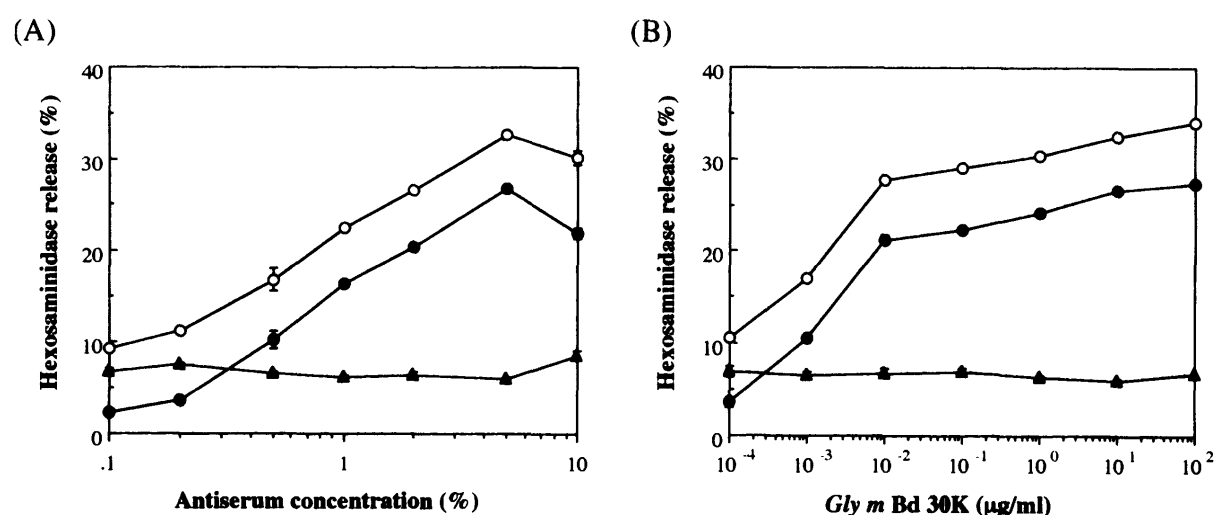


Fig. 5. Effect of the Antiserum or Antigen Concentration on β -*N*-Acetylhexosaminidase Released from the RBL-2H3 Cells.

The β -*N*-acetylhexosaminidase release is expressed as a percentage of the total enzyme activity contained in a lysate of 1×10^5 cells. Data are means $\pm$ SD ($n=3$). A: Cells were pre-incubated with the anti-*Glym* Bd 30K serum at given concentrations. The cells were then stimulated by a buffer containing 10 μ g/ml of *Glym* Bd 30K (open circles), or by the buffer alone (solid triangles). β -*N*-Acetylhexosaminidase released by the allergen was obtained by subtracting the latter from the former (solid circles). B: Cells preincubated with a medium containing 5% of the anti-*Glym* Bd 30K serum (open circles) or 5% of the non-immunized serum (solid triangles) were stimulated with *Glym* Bd 30K at given concentrations. β -*N*-Acetylhexosaminidase release mediated by the antiserum was obtained by subtracting the latter from the former (solid circles).

proteins were subcutaneously injected into BALB/c mice with an alum adjuvant, IgE recognizing KSTI was preferentially produced,¹⁶⁾ while IgE recognizing *Glym* Bd 30K could not be detected in the antiserum. These facts suggest that the production of IgE recognizing KSTI would take precedence over that recognizing *Glym* Bd 30K when both proteins were simultaneously immunized. Thus, KSTI is considered to be the most potent experimental allergen among soyproteins toward BALB/c mice.

The role of IgG in the type-I allergic reaction is still unclear. Human IgG4, comparable to mouse IgG1, has been discussed on the one hand as an anaphylactic antibody, and on the other hand as blocking antibody against anaphylaxis.¹⁷⁾ IgG1 in mice has been suggested to be able to exhibit reaginic activity in the PCA reaction.¹⁴⁾ It has also been reported that the cross-linking of IgG receptors on RBL-2H3 cells could not induce histamine release,

whereas the binding capacity for IgG of RBL-2H3 cells was reported¹⁸⁾ and the Fc γ type of receptor for IgG was cloned from a cDNA library of the cells.¹⁹⁾ The anti-*Glym* Bd 30K serum contained all IgG subclasses including IgG1; however, the removal of IgG had no effect on the *in vitro* assay with RBL-2H3 cells nor on the *in vivo* PCA reaction. On the contrary, removal or denaturation of IgE resulted in striking damage to the reaginic activity of the antiserum. These results show that IgG specific for *Glym* Bd 30K might not exhibit reaginic activity, and that almost all the reaginic activity is attributable to IgE specific for *Glym* Bd 30K, which suggests that the assay method with the antiserum could be a simulation of the IgE-mediated allergy in humans.

The *in vitro* assay with RBL-2H3 cells reveals the allergenicity arising the activation of the basophils or mast cells *via* IgE binding to Fc ϵ RI located on the cell surface.

The sensitivity of this assay is no less than that of the PCA reaction. The technique for this assay method is not complicated, and the combination of the allergen and antibody can be altered easily. Thus, the assay using the anti-*Glym* Bd 30K serum could be a suitable and useful simulator of allergic patients for detecting the allergenicity of soybean products, especially in the course of development of hypoallergenic soy products. Furthermore, the assay may aid in studying the epitopes of *Glym* Bd 30K for IgE. In this way, the assay method we have developed may provide useful knowledge to solve the allergy evoked by soyprotein intake.

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