

Tomato endo beta-mannanase: A candidate of potential tomato allergen protein detected with human monoclonal antibody established from a patient suffered from Japanese cedar pollinosis

Soichiro Endo, Takahiro Yamada, Takayuki Kobayashi, Yasunori Naganawa, Toshihiro Tabuchi and Hiroshi Shinmoto*

Graduate School of Natural Resources and Biology, Tamagawa University, Machida, Tokyo, Japan

Abstract. Peripheral blood lymphocytes from a patient allergic to Japanese cedar pollens were transformed by Epstein-Barr virus infection. Some transformed B-lymphoblastoid cells (BLCs) secreted IgM class antibodies to cedar pollen extracts and tomato fruit extracts. One stable human-mouse hybridoma clone Y-22-3-3 secreting IgM class monoclonal antibody to tomato fruit extracts was established by cell fusion of BLCs with mouse myeloma cells. Western blot analysis of tomato extracts showed Y-22-3-3 monoclonal antibody recognized a tomato protein with a molecular weight of 40 kDa. The CBB-stained 40 kDa protein from antibody-affinity chromatography was analyzed by MALDI-TOF/TOF, and identified as tomato endo-beta-mannanase, which was previously reported as one of the potential candidates for tomato allergens.

Keywords: Japanese cedar pollen, tomato, allergy, monoclonal antibody, hybridoma

1. Introduction

Japanese cedar (*Cryptomeria japonica*) trees spread pollens over most areas of Japan from late January to April, and cause severe pollen allergy (Japanese cedar pollinosis) [1–4]. Major Japanese cedar pollen allergens, Cry j1 and Cry j2, were identified with sera from patients, and cDNAs of the allergens were cloned [5–7].

Patients allergic to Japanese cedar pollens sometime have oral allergy syndrome to tomato fruits [8]. Those cedar pollen proteins and tomato allergen proteins could have common allergen epitopes. Because tomato oral allergy syndrome related allergens seemed

to be heat labile, common allergens might have conformational epitopes that lose allergenicity by heating or chemical treatment such as reduction of disulfide bonds with 2-mercaptoethanol.

In this study, we generated a human-mouse hybridoma secreting human monoclonal antibody capable of binding tomato fruit extracts by transformation of B-lymphocytes from an allergic patient followed by cell fusion with mouse myeloma cells. By using the human monoclonal antibody, we identified a tomato enzyme protein as a candidate of a potential tomato allergen.

2. Material and methods

2.1. Preparation of tomato extracts as allergens

Twelve commercial tomato cultivars were purchased

*Corresponding author: Hiroshi Shinmoto, Graduate School of Natural Resources and Biology, Tamagawa University, 6-1-1 Tamagawa-gakuen, Machida, Tokyo 194-8610, Japan. Tel./Fax: +81 42 739 8267; E-mail: shnmt920@agr.tamagawa.ac.jp.

at a local market and fifteen wild tomato varieties were grown in our green house. Tomato fruits were homogenized with food processor for 5 min, and homogenates were centrifuged at $15,000 \times g$ for 20 min. The supernatants were used as tomato extracts. The same volumes of supernatants from ten commercial cultivars were mixed and used as tomato extracts (tomato allergen) for ELISA.

2.2. Enzyme-linked immunosorbent assay (ELISA)

Allergen solutions (tomato extracts) were diluted with 0.05 mol/L NaHCO_3 (protein concentration of 0.0001–0.001 mg/mL), 0.06 mL of the solution was pipetted into 96-well ELISA plates, and the plates were kept at 4°C for 16 h. After the allergen solution was discarded, the plates were blocked with a blocking reagent (Block Ace, DS Pharma Biochemical Co. Ltd., Japan) at room temperature for 1 h. The plates were then washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBS-Tween). The culture supernatants of BLCs (0.05 mL) were pipetted into the antigen-coated wells and incubated at room temperature for 1 h. The plates were then washed three times, and 0.05 mL of antibodies (anti-human IgA, IgG, IgM, and IgE, 1/2,000 dilution, Biosource International, USA) conjugated with horseradish peroxidase (HRPO) were added to the plates. After 1 h of incubation at room temperature, the plates were washed six times and peroxidase activities bound on the plates were measured by adding 0.1 mL of substrate solution (0.3 mg/mL 2,2'-azino-di-(3-ethyl benzthiazolin sulfonic acid) (ABTS), 0.03 % H_2O_2 in 0.1 mol/L citrate buffer pH 4.0).

2.3. Generation of a human-mouse hybridoma secreting IgM-class anti-tomato fruit human monoclonal antibody

From our Epstein-Barr virus-transformed human B-lymphoblastoid cell (BLC) library [9–11], BLC #Y213 secreting IgM-class anti-tomato fruit antibody from a patient suffered from Japanese cedar pollinosis was chosen for generation of human-mouse hybridomas [12–15].

An 8-azaguanine-resistant (HAT sensitive) mouse myeloma cell line, SP2/O, was cultured in an ERDF medium (Kyokuto Pharmaceutical Industrial, Japan) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, Japan) and 0.05 mmol/L ouabain to obtain an ouabain-resistant cell line, SP2/O3. BLC #Y213 cells

(2×10^7) and SP2/O3 cells (2×10^7) were fused with 50% (v/v) polyethyleneglycol (Sigma-Aldrich, Japan). The fused cells were then plated into three 96-well microculture plates. Hybridoma cells were grown in O-HAT medium (the ERDF medium supplemented with 10% FCS, 0.1 mmol/L hypoxanthine, 0.0004 mmol/L aminopterin, 0.016 mmol/L thymidine, and 0.0002 mmol/L ouabain) [12–14]. Antibody-producing hybridomas were cloned by limiting dilution method. Finally, the established clone secreting IgM-class anti-tomato fruit antibody was named as Y-22-3-3.

2.4. Western blot analysis of tomato allergen with Y-22-3-3 monoclonal antibody

Extracts from wild tomato #33 was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10–20% polyacrylamide gradient gel, Oriental Instruments, Japan). The extract was treated SDS-sample buffer with or without 2-mercaptoethanol at 37°C or 95°C for 5 min. After PAGE, separated proteins were transferred from the gel to PVDF membrane (ATTO Products, Japan) electrically. After the membrane was blocked with skimmed milk solution (MEG-MILK SNOW BRAND Co., Ltd., Japan), Y-22-3-3 monoclonal antibody was reacted followed by reaction with HRPO conjugated anti-human IgM antibodies. Finally, the membrane was developed with the chemical luminescence substrate (ImmunoStar Zeta, Fuji-film Wako pure chemical, Japan) and recorded with CCD-photo LumiCube device (Liponics, Japan).

2.5. Antibody-affinity isolation of tomato protein with MEP affinity chromatography

To isolate a protein recognized with Y-22-3-3 monoclonal antibody, MEP (4-mercaptoethyl pyridine) HyperCel resin (PALL Biosepra, France) affinity chromatography was done. The MEP ligand has an affinity for immunoglobulins including IgA, IgE, IgM, and IgG. At first, 1 mL of Y-22-3-3 antibody solution (culture supernatants of Y-22-3-3 hybridoma) was charged onto small MEP resin column (0.015 mL) equilibrated with Tris-HCl buffer (0.05 mol/L, PH 8.2). Then the column was washed with Tris-HCl buffer, and 0.2 mL of tomato extracts was reacted. After washing the column, proteins bound onto the column were eluted with acetate buffer (0.05 mol/L PH 3.0).

2.6. MALDI-TOF/TOF mass spectrometric analysis of a tomato protein

After the SDS-PAGE of MEP-eluted protein at the non-reduced condition, gel piece of CBB-stained protein spot of Fig. 1 (CBB, 2ME-, *) was washed with acetonitrile (50%) and vacuum dried. Then the gel piece was digested with trypsin at 37°C for 10 hr. Trypsinized samples were analyzed using the Bruker Autoflex Speed with Lift ion optics (MALDI-TOF/TOF analyzer, WI, USA) [16]. MS/MS-obtained ion searches were performed with the program “Mas-cot” for in-house use.

3. Results and discussion

From more than 5,000 oligo-clone library of BLCs, BLC #Y213 secreting IgM class antibody to tomato extracts was chosen for further experiments. Because of the instability of antibody secretion of BCLs, Y213 cells were fused with mouse myeloma SP2/O3 cells to obtain stable human-mouse hybridomas. Finally, we established a hybridoma clone Y-22-3-3 secreting IgM class human monoclonal antibody to tomato extracts.

Binding of Y-22-3-3 monoclonal antibody to extracts from 12 commercial tomato cultivars and 15 wild tomato varieties was analyzed by ELISA. Y-22-3-3 monoclonal antibody bound to the all the extracts from commercial tomato cultivars almost equally. In the case of wild tomato varieties, Y-22-3-3 monoclonal antibody strongly bound to extract from #33 and weakly bound to extracts from #24, 26, 29, and 30 (data not shown).

To identify the protein recognized by Y-22-3-3 monoclonal antibody, the extract from wild tomato #33 was subjected to Western blot analysis. The extract was treated with SDS-sample buffer with or without 2-mercaptoethanol at 37°C or 95°C. As shown in Fig. 1 (Western), an antibody recognized band was observed at 40 kDa at the non-reduced conditions (37°C and 95°C). In the presence of the 2-mercaptoethanol, less signal was shown with the extract treated at 37°C. The extract heated at 95°C with 2-mercaptoethanol showed very weak signal for antibody binding. Those results suggested that the protein recognized with Y-22-3-3 monoclonal antibody might have a conformation-dependent epitope that could be hidden with the reduction of disulfide bonds of cystine by 2-mercaptoethanol treatment.

To isolate tomato protein recognized with Y-22-3-3 monoclonal antibody, MEP (4-mercaptoethyl pyridine)

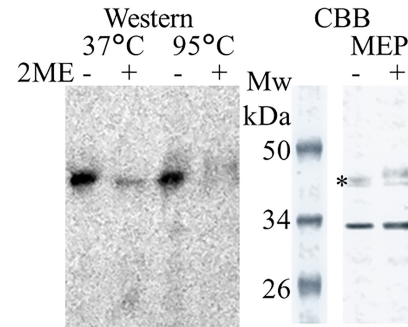


Fig. 1. Western blot analysis of extracts from wild tomato #33 with Y-22-3-3 human monoclonal antibody. Tomato extracts were treated with SDS-sample buffer with or without 2-mercaptoethanol (2ME) at 37°C or 95°C and reacted with Y-22-3-3 human monoclonal antibody (Western). Allergen candidate protein was isolated by MEP-IgM affinity chromatography and CBB-stained band with the molecular weight of 40 kDa (CBB, 2ME-, *) on SDS-PAGE gel was used for further analyzed by MALDI-TOF/TOF mass spectrometry.

- 1 R.VILLQSLR.L*
- 2 K.QYVEWAVQR.G*
- 3 -.MNNRAYKAQR.E
- 4 K.RRPSLEQLLR.M
- 5 MPQRNPFPPER
- 6 R.ALLGCTSSSAFAGTCTR.R
- 7 MAGVGPSVDRTDKASVGQEAR
- 8 SFQLAFSLRNISLNEGGEPR
- 9 K.VAYKDDPTILSWELINEPR.C*
- 10 K.SIDSNHLLIEIGLEGFYGNDR.Q*

Fig. 2. Peptides sequences of trypsinized tomato 40 kDa protein. Gel piece of CBB-stained protein spot of Fig. 1-CBB* was digested with trypsin. Trypsinized samples were analyzed by MALDI-TOF/TOF mass spectrometry.

1 FSNNFVYTD GTHFALNGKS LYINGFNAYW LMYIADPST RIKVTNTFQQ
 51 ASKYKMNVAR TWAFSHGGSR PLQSAPGVYN EQMFQGLDFV ISEAKYGIH
 101 LIMSLVNNWD AFGGKKQYVE WAVQRGQKLT SDDDFFTNPM VKGFYKNNVK
 151 VVLTRVNTIT K VAYKDDPTI LSWELINEPR CPSDLSGKTF QNWLVMAGY
 201 LKSIDSNHLL EIGLEGFYGN DMRQYNPNYS IFGTNFISNN QVQGIDFTTI
 251 HMYPNQWLPG LTQEAQDKWA SQWIVQHIDD SKMLKKPLLI AEFKSTKTP
 301 GYTVAKRDNV FEKIYGTIFN CAKSGGPGCG GLFWQLVQGG MSSFDGQYQ
 351 VLQESPSTSR VILLQSLRLS KLS

Fig. 3. Amino acid sequence of tomato endo-beta-mannanase. Amino acid sequences underlined are #1, #2, #9, and #10 peptide in the Fig. 2.

HyperCel resin affinity chromatography was done. The eluted proteins from MEP-IgM-tomato extract affinity chromatography were separated by SDS-PAGE (Fig. 1-CBB). CBB-stained band with the molecular weight of 40 kDa was subjected to MALDI-TOF/TOF mass spectrometric analysis. Unknown protein band with the molecular weight of 33 kDa was not analyzed.

Amino acid sequences of ten peptides from trypsin digestion of the CBB-40 kDa band were shown in Fig. 2. Of those peptides, four peptides (#1, #2, #9, and #10) were found to be included in the amino acid sequence of tomato endo-beta-mannanase (gi|66360069) chain A as shown in Fig. 3.

In 2013, Welter et al. [16] reported five new allergen candidate proteins from tomato fruits by 2-D immunoblots with IgE from tomato allergy patients. One of those proteins was reported to be tomato endo-beta-mannanase. Our human IgM-class Y-22-3-3 monoclonal antibody from a patient suffered from cedar pollen allergy also recognized tomato endo-beta-mannanase. The results obtained in this study strongly suggested the tomato endo-beta-mannanase may have an important role on the tomato allergy including oral allergy syndrome.

References

- [1] T. Okawa, A. Konno, T. Yamakoshi, T. Numata, N. Terada and M. Shima, Analysis of natural history of Japanese cedar pollenosis, *Int Arc Allergy Immunol* **131** (2003), 39–45.
- [2] M. Okuda, Epidemiology of Japanese cedar pollenosis throughout Japan, *Ann Allergy Asthma Immunol* **91** (2003), 288–296.
- [3] Q. Wang, S. Nakamura, S. Lu, G. Xiu, D. Nakajima, M. Suzuki, K. Sakamoto and M. Miwa, Release behavior of small sized daughter allergens from *Cryptomeria japonica* pollen grains during urban rainfall event, *Aerobiologia* **28** (2012), 71–81.
- [4] T. Fujimura and S. Kawamoto, Spectrum of allergens for Japanese cedar pollinosis and impact of component-resolved diagnosis on allergen-specific immunotherapy, *Allergology International* **64** (2015), 312–320.
- [5] T. Sone, N. Komiyama, K. Shimizu, T. Kusakabe, K. Morikubo and K. Kino, Cloning and sequencing of cDNA coding for Cry j1, a major allergen of Japanese cedar pollen, *Biochem Biophys Res Commun* **199** (1994), 619–625.
- [6] N. Komiyama, T. Sone, K. Shimizu, K. Morikubo and K. Kino, cDNA cloning and expression of Cry j2 the second major allergen of Japanese cedar pollen, *Biochem Biophys Res Commun* **201** (1994), 1021–1028.
- [7] T. Ohtsuki, Y. Taniguchi, K. Kohno, S. Fukuda, M. Usui and M. Kurimoto, Cry j2, a major allergen of Japanese cedar pollen, shows polymethylgalacturonase activity, *Allergy* **50** (1995), 483–488.
- [8] Y. Kondo, R. Tokuda, A. Urisu and T. Matsuda, Assessment of cross-reactivity between Japanese cedar (*Cryptomeria japonica*) pollen and tomato fruit extracts by RAST inhibition and immunoblot inhibition, *Clinical & Experimental Allergy* **32** (2002), 590–594.
- [9] N.A. Brown and G. Miller, Immunoglobulin expression by human B lymphocytes clonally transformed by Epstein Barr virus, *J Immunol* **128** (1982), 24–29.
- [10] H. Shinmoto, K. Nakahara, M. Kobori and T. Tsushida, Preparation of human immortalized B-cells secreting antibodies against food allergens, *Biotechnology Techniques* **12** (1998), 545–547.
- [11] H. Shinmoto, K. Yamagishi, T. Kimura, M. Suzuki and Y. Sakai, Antigenicity estimation of collagenase-treated gelatins by a novel assay system using human antibodies secreted by B-lymphoblastoid cells, *Food Sci Technol Res* **7** (2001), 331–332.
- [12] S. Chiba, H. Yokoyama, A. Kumazawa, M. Shimmoto, Y. Naganawa and H. Shinmoto, Epitope analysis of Japanese cedar pollen allergen Cry j2 with a human IgM class monoclonal antibody 404-117, *Human Antibodies* **25** (2017), 17–21.
- [13] Y. Naganawa, H. Shinmoto and M. Shimmoto, Generation of mouse-human hybridomas secreting human monoclonal antibodies to Japanese cedar pollen allergen Cry j1, *Human Antibodies* **14** (2005), 27–31.
- [14] Y. Naganawa, M. Takeda, M. Shimmoto and H. Shinmoto, Epitope analysis of Japanese cedar pollen allergen Cry j1 with the human monoclonal antibody 4701-1, *Human Antibodies* **22** (2013), 73–76.
- [15] H. Shinmoto, Y. Naganawa, M. Shimmoto and S.J. Maleki, Generation of mouse-human hybridomas secreting antibodies against peanut allergen Ara h1, *Cytotechnology* **46** (2004), 19–23.
- [16] S. Welter, K. Lehmann, S. Dölle, D. Schwarz, W. Weckwerth, C. Scheler, M. Worm and P. Franken, Identification of putative new tomato allergens and differential interaction with IgEs of tomato allergic subjects, *Clinical & Experimental Allergy* **43** (2013), 1419–1427.