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Original Article

Nattokinase, a subtilisin family serine protease, is a novel allergen contained in the traditional Japanese fermented food natto

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PGA polygamma glutamic acid

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ABSTRACT

Background: Immediate allergy caused by natto, a popular Japanese food prepared by fermenting soybeans with *Bacillus subtilis* var. *natto*, has been reported. Polygamma glutamic acid (PGA) in the sticky substance around natto beans has been reported to be a causative allergen of natto allergy. However, some of our patients with natto allergy were negative for PGA in the skin prick test (SPT). The sticky substance of natto beans contains a subtilisin family serine protease, nattokinase, along with PGA. In this study, we aimed to examine the antigenicity of nattokinase in natto allergy.

Methods: Eight patients, who developed symptoms after ingesting natto and positively reacted to natto (seven to the sticky substance in natto and one to the whole natto product) in their SPT, were enrolled in this study. To analyze IgE reactivity, we performed immunoblotting, ELISA, and SPT for natto (bean and sticky substance), and/or PGA, and/or nattokinase and/or cultured *B. subtilis* var. *natto* extract.

Results: In the SPT, four cases each were PGA-positive and PGA-negative. Immunoblotting of the sera from PGA-negative patients showed a protein band at 30 kDa, which was identified as nattokinase. Three PGA-negative cases, but not three PGA-positive cases, showed a positive reaction to nattokinase in the SPT and had a history of atopic dermatitis. The ELISA for nattokinase revealed a positive reaction of PGA-negative cases and negative reaction of PGA-positive cases in the SPT.

Conclusions: We identified a subtilisin family serine protease, nattokinase, as a novel allergen in natto allergy patients unsensitized to PGA.

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Introduction

Natto is a Japanese food produced by fermenting soybeans with *Bacillus* (*B.*) *subtilis* var. *natto*. Natto soybeans have a sticky substance around them. This sticky substance contains polygamma glutamic acid (PGA) and nattokinase. Nattokinase is an enzyme produced by *B. subtilis* var. *natto*, and it was discovered by Sumi *et al.* in 1987.^{1,2} The molecular weight of nattokinase is approximately 27.7 kDa; it consists of 275 amino acid residues.^{3,4} Contrary to its name, nattokinase is a subtilisin family serine protease and has no kinase activity.

Natto allergy is an immediate allergy mediated by IgE and shows positive results in the skin prick test (SPT),^{5–22} ELISA,²⁰ and basophil activation test for natto.²² Inomata *et al.* reported that the causative allergen of natto allergy was PGA.¹² Until 2021, all

reported cases of natto allergy showed a positive reaction in the SPT for PGA.^{12,14,17,19,20} Engaging in marine sports is considered a risk factor for natto allergy because 12 of 13 patients with natto allergy (92%) have a history of engaging in marine sports.¹⁸ These individuals may become sensitized to PGA via contact with marine sources such as the tentacles of jellyfish.¹³

However, we encountered four cases of natto allergy with a positive reaction to the sticky substance of natto and a negative reaction to PGA in the SPT. In immunological assays/evaluations, the sera of these patients showed positive reactions to natto but not to PGA. Furthermore, they did not have a history of engagement in marine sports. In this study, we examined the antigenicity of nattokinase of natto.

Methods

Patients

We diagnosed patients to have natto allergy based on clinical symptoms after ingesting natto and a positive reaction to natto in

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Table 1
Characteristics of the patients.

Group	No.	Age (years)/sex	Marine sports	Atopic dermatitis	Asthma	Pollinosis	Total IgE (IU/mL)	Onset time after ingesting natto	Symptoms after ingesting natto
PGA-negative	1	54/F	Yes, Surfing	Yes	Yes	Yes	2376	1–2 h	Discomfort and swelling in the throat; no urticaria; no respiratory distress
	2	31/M	No	Yes	Yes	Yes	453	10–15 min	Swelling of the throat and lips; no urticaria; no respiratory distress
	3	33/F	No	Yes	Yes	No	3521	Immediately	Dyspnea and tightness in the throat; no urticaria
	4	20/F	No	Yes	No	Yes	1472.3	20 min–1 h	Itching in the throat and difficulty breathing; nausea; no urticaria
PGA-positive	5	27/F	Yes, Surfing	No	No	Yes	179	2–3 h	Urticaria; dyspnea
	6	41/M	Yes, Surfing	No	No	Yes	612.1	30 min	Urticaria; abdominal pain; diarrhea; vomiting; decreased consciousness
	7	21/F	No	No	No	Yes	291.6	3–20 h	Urticaria; loss of consciousness; decreased blood pressure
	8	17/M	No	No	No	Yes	374.6	Approximately 10 h	Urticaria

F, female; M, male; h, hours; min, minutes; PGA, polygamma glutamic acid.

the SPT. Among the patients with natto allergy at our hospital from 2018 to 2020, eight patients (three males and five females; age, 17–54 years) who agreed to undergo allergen analysis were enrolled in this study (Table 1). Two cases previously reported¹⁶ to be PGA-positive in the SPT were included and named as cases 5 and 6.

The patients were divided into two groups, PGA-positive and PGA-negative, based on the results of the PGA SPT. Healthy control participants, two males and two females aged 38–52 years, without natto allergy (based on interviews) were included.

Sample preparation

PGA (molecular weight $\geq 750,000$ Da; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in physiological saline (0.01–100 mg/mL). Commercially available natto (Mizkan Holdings, Aichi, Japan) (10 g) was added to 10 mL of ultrapure water and mixed by agitation for 1 h at 4 °C. The centrifugal supernatant was used as the “sticky sample” (the major component was PGA). The precipitated beans were collected; 1 g of beans was mixed with 1 mL of purified water and used as the “bean sample.” Nattokinase (Wako Pure Chemical, Osaka, Japan) was dissolved in physiological saline (0.01–1 mg/mL). *B. subtilis* var. *natto* (Takahashi strain; Takahashi Yuzo Laboratory, Yamagata, Japan) was used as is or after culturing. In the latter, the bacterial cells were cultured on trypticase soy agar medium at 32 °C for 24 h. Protein was extracted using buffer containing 7 M urea, 2 M thiourea, and 30 mM Tris–HCl (pH 8.5).

SDS-PAGE

The proteins extracted from the bean, sticky, and cultured *B. subtilis* var. *natto* were subjected to SDS-PAGE (Novex® NuPAGE® 4–12% Bis-Tris Gel with NuPAGE® MOPS SDS Running Buffer; Thermo Fisher Scientific, Waltham, MA, USA) under a reducing condition. The proteins were stained with SimplyBlue™ SafeStain (Thermo Fisher Scientific), and the staining results were imaged using Typhoon FLA 9500 (GE Healthcare, Little Chalfont, UK).

Two-dimensional PAGE (2D-PAGE)

Proteins (50 µg) from the sticky and cultured *B. subtilis* var. *natto* were dissolved in Destreak Rehydration solution (GE Healthcare) containing a reducing agent. These samples were separated using 2D-PAGE (7 cm Immobiline DryStrip pH 3–10 NL; GE Healthcare and Novex® NuPAGE® 4–12% Bis-Tris Gel with NuPAGE® MOPS SDS Running Buffer). The proteins were stained with SyproRuby (Thermo Fisher Scientific), and the fluorescence images were recorded using Typhoon FLA 9500.

Immunoblotting

The proteins separated using SDS-PAGE or 2D-PAGE were transferred onto polyvinylidene difluoride membranes (Immobilon-FL PVDF; Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBS-T) for 1 h at approximately 22 °C. The membranes were then incubated in 5% serum diluted in 5% skim milk and PBS-T for 1 h at approximately 22 °C, followed by incubation with 0.2 µg/mL antihuman IgE antibody-horseradish peroxidase conjugate (SeraCare, Milford, MA, USA) in 5% skim milk in PBS-T for 1 h at approximately 22 °C. The chemiluminescence reaction was developed using ECL Plus western blotting substrate (Thermo Fisher Scientific). Fluorescent images were recorded using Typhoon FLA 9500. For immunoblotting inhibition assay, nattokinase was preheated at 95 °C for 1 min and diluted to 0, 0.01, 0.1, and 1.0 mg/mL with 5% serum diluted with 5% skim milk in PBS-T. After incubating the samples for 1 h at approximately 22 °C, immunoblotting was performed.

Protein identification

Image data obtained following 2D-PAGE and 2D-WB were compared using SameSpots software (SCRUM, Tokyo, Japan) to select protein spots with the same molecular weight and isoelectric point. The relevant IgE antibody-bound protein spots were excised from the PVDF membrane (2D-PAGE samples) and digested using Trypsin Gold, Mass Spectrometry Grade (Promega, Madison, WI, USA). The peptides were analyzed using an Eksigent ekspert™ nano LC 400 system (Sciex, Framingham, MA, USA) equipped with a C18 reverse-phase column (Nano cHiPLC column 75 µm × 15 cm ChromXP C18-CL 3 µm 120 Å, Sciex), which was coupled to TripleTOF™ 6600 (Sciex) equipped with a nano-electrospray ionization source (NanoSpray™-III, Sciex). Tandem mass spectrometry (MS/MS) spectra were analyzed using Protein Pilot™ software 5.0 (Sciex) against *B. subtilis* var. *natto* data from the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov/>). Proteins were identified based on two or more peptides with a confidence of >95%, number of unique peptides detected, and sequence coverage.

ELISA

Nattokinase (20 µg) and protein from the cultured *B. subtilis* var. *natto* (10 µg) were immobilized in each well of a Nunc MaxiSorp flat-bottom 96-well plate (Thermo Fisher Scientific). Diluted serum (10%) in 1% skim milk in PBS-T was added to the wells, and the plate was incubated for 1 h at approximately 22 °C. The samples were

incubated with 0.2 µg/mL antihuman IgE antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD, USA) in 1% skim milk in PBS-T for 1 h at approximately 22 °C. The colorimetric reaction was developed using a 1-Step Ultra TMB-ELISA kit (Thermo Fisher Scientific); the reaction was terminated by adding 2 M H₂SO₄. The absorbance of the samples was measured at 450 nm using a multi-plate optical densitometer (EnSpire™ Multimode Plate Reader; PerkinElmer, Waltham, MA, USA). The cut-off value was determined as the average value obtained from healthy control subjects +5 SD.

SPT

The SPT was performed on the patient's forearm using a SmartPractice prick lancet (SmartPractice, Canada) with natto (bean and sticky substances), PGA, and nattokinase. Nattokinase was tested in six patients. Histamine dihydrochloride (10 mg/mL; Torii, Tokyo, Japan) was used as the positive control and saline was used as the negative control. A reaction with a wheal diameter of one-half or more of that of histamine dihydrochloride was considered a positive result in the SPT.

Ethics

The study was approved by the ethics committee of Fujita Health University (approval no. HM20-204). Written informed consent was obtained from all participants, and patient anonymity was preserved using methods approved by the Ethics Committee. This study was performed in accordance with the tenets of the Declaration of Helsinki.

Results

Characteristics of the patients

Among the eight patients, four were PGA-positive and four were PGA-negative in the SPT. Only three of the eight patients (37.5%) had a history of surfing. None of the PGA-positive patients had a history of atopic dermatitis, whereas all PGA-negative patients had a history of atopic dermatitis. After ingesting natto, all PGA-positive patients experienced general urticaria, whereas none of the PGA-negative patients experienced urticaria. Symptoms in the PGA-negative cases were localized to the pharynx and larynx (Table 1).

Specific IgE antibody for natto and cultured *B. subtilis* var. *natto* detected using immunoblotting

One of the four PGA-positive cases and all four PGA-negative cases showed binding of the IgE antibody to the sticky sample of natto and cultured *B. subtilis* var. *natto*. The molecular weight of the reactive protein was approximately 30 kDa. Therefore, a protein of approximately 30 kDa common to the sticky sample and cultured *B. subtilis* var. *natto* was predicted as the causative allergen (Fig. 1a). We performed immunoblotting inhibition assay with nattokinase against natto beans and sticky sample in cases 1–4 (PGA-negative group). In the PGA-negative group, IgE binding was inhibited in a nattokinase concentration-dependent manner (Fig. 1b).

Identification of a 30-kDa protein using 2D-WB and MS/MS analyses

Based on the MS/MS analysis using the *B. subtilis* var. *natto* database of the IgE-bound allergens from PGA-negative patients who showed a reaction to both sticky sample and cultured *B. subtilis* var. *natto* in 2D-WB, the 30-kDa protein was identified as nattokinase (accession #: 5GL8_B, identified peptides (confidence

>95%); 35, sequence coverage; 71.6%). The 30-kDa protein, which yielded a positive reaction in one of the PGA-positive cases, was not nattokinase but rather the biofilm matrix protein TasA (accession #: WP_014480250.1, identified peptides (confidence >95%); 17, sequence coverage; 52.1%) (Fig. 2). As we performed MS/MS analysis using data from the *B. subtilis* var. *natto* database, proteins of other species were not identified. However, the results of mass spectrometry of TasA showed a high sequence coverage of 52.1%; therefore, we considered that TasA is likely a protein derived from *B. subtilis* var. *natto*.

Antigenicity of nattokinase determined using ELISA and SPT

Serum IgE was positive for nattokinase in all PGA-negative cases and negative in all PGA-positive cases (Table 2, Fig. 3). The SPT for nattokinase was performed in six of the eight cases: three PGA-positive and three PGA-negative cases. All three PGA-negative cases showed a positive reaction to nattokinase, whereas all three PGA-positive cases showed no reaction to nattokinase (Table 2).

Discussion

PGA is considered the causative allergen of natto allergy.¹² However, we encountered four cases of natto allergy with a negative reaction to PGA and sticky substance of natto in the SPT. As nattokinase is present along with PGA in the sticky substance of natto,^{1,2} we identified the allergenicity of nattokinase in the patients with PGA-negative natto allergy using immunoblotting, ELISA, and SPT. The sera of these patients responded to nattokinase in the ELISA. In immunoblotting and immunoblotting inhibition assay, the bands of the bean of natto and sticky substance of natto in the PGA-negative group were confirmed to be nattokinase. In the SPT of these patients, three patients showed a positive reaction to nattokinase and one patient reacted to cultured *B. subtilis* var. *natto* extract, which mainly contained nattokinase. Therefore, nattokinase was confirmed as an allergen in the cases with PGA-negative natto allergy.

As mentioned above, the sticky substance of natto includes PGA and nattokinase. In the fermentation process, nattokinase is a primary metabolite and PGA is a secondary metabolite.^{23,24} PGA is produced as a nutrient source when cells proliferate and starve. Therefore, PGA is produced after the cessation of cell proliferation during the fermentation process.²⁴ We performed immunoblotting using sera of PGA-negative/nattokinase-sensitized patients to confirm if uncultured *B. subtilis* var. *natto* contained nattokinase. From the results, it was considered that the uncultured *B. subtilis* var. *natto* did not contain sufficient nattokinase to detect IgE antibody (data not shown). In order to confirm whether the cultured *B. subtilis* var. *natto* used did not contain PGA, ELISA was performed using the sera of PGA-positive patients. The results revealed that the cultured *B. subtilis* var. *natto* extract used in this study did not contain enough PGA to be detected by IgE antibodies (data not shown). Inomata *et al.*⁹ also reported that PGA-positive natto allergy cases do not react to *B. subtilis* var. *natto* powder in the SPT, but they did not provide detailed information on how to prepare the *B. subtilis* var. *natto* powder used in their SPT in the report.

Awatani-Yoshidome *et al.* also reported a natto allergy case that showed a negative reaction to PGA and positive reaction to nattokinase in SPT.²⁵ Therefore, in addition to PGA, nattokinase is a novel allergen mediating natto allergy.

Accordingly, natto allergy cases were grouped into PGA- and nattokinase-sensitized groups. We evaluated patients in a single hospital, which is a limitation of this study. It is generally difficult to analyze the allergen causing natto allergy because only a few

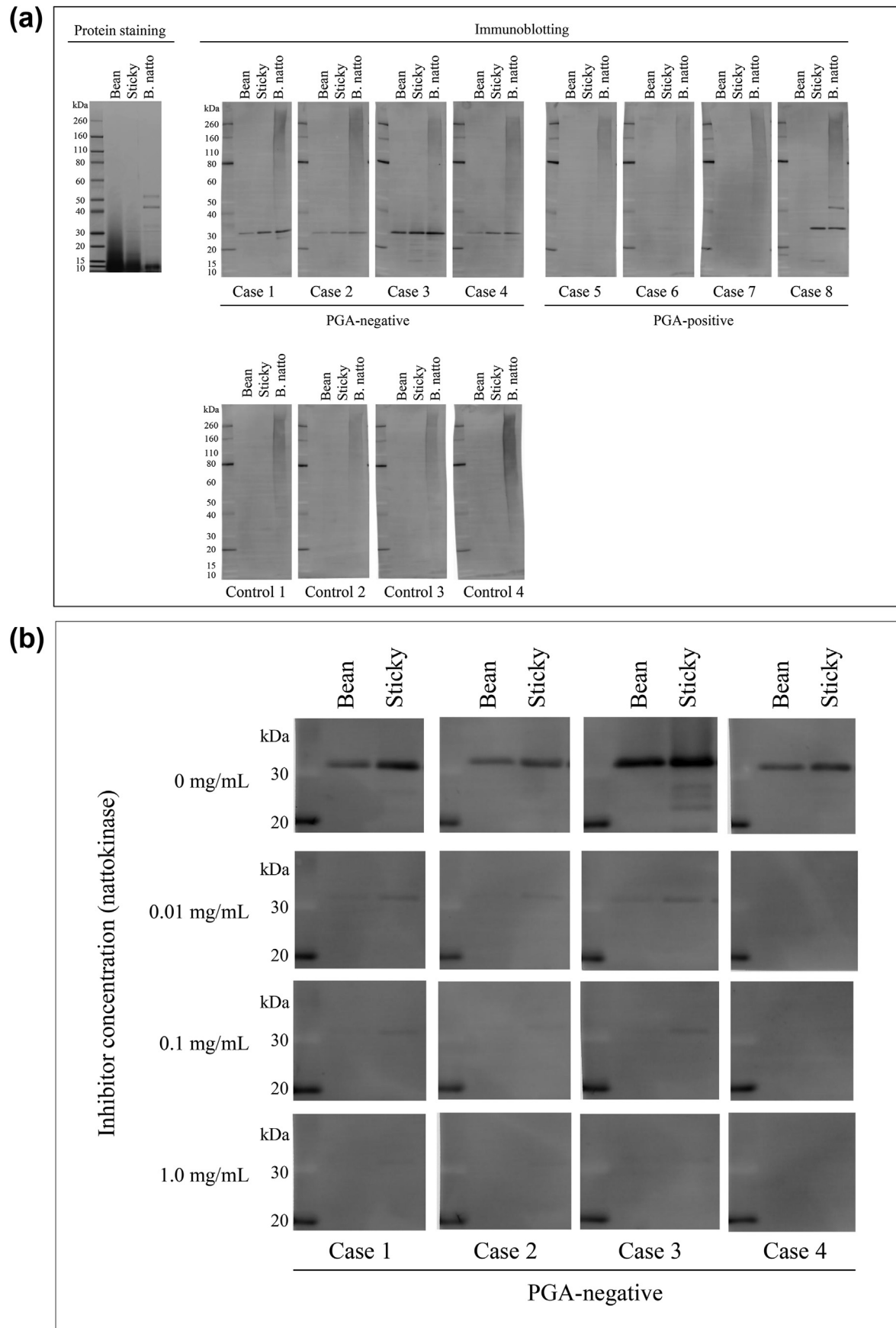


Fig. 1. All PGA-negative cases were sensitized to an approximately 30-kDa protein, nattokinase, in the natto product (bean and sticky samples) and cultured *B. subtilis* var. *natto*. The sensitizing antigens were evaluated by immunoblotting (a), and inhibition experiments confirmed that it was nattokinase (b). Bean, bean sample of natto; Sticky, sticky sample of natto; B. natto, cultured *Bacillus subtilis* var. *natto* (Yuzo Takahashi Laboratory, Yamagata, Japan); Control, healthy control subjects.

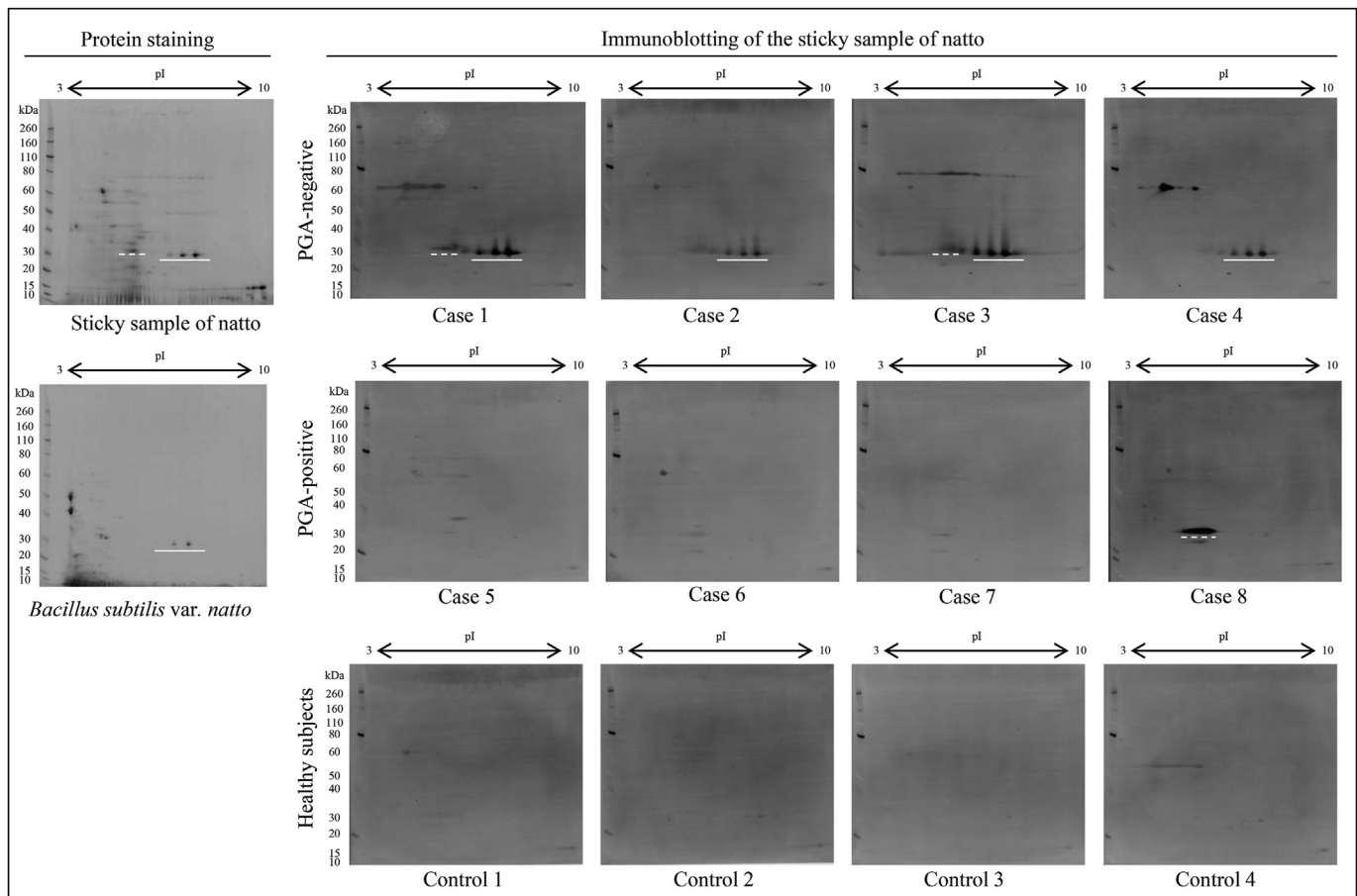


Fig. 2. Results of two-dimensional PAGE (2D-PAGE) of the sticky sample and protein extracted from cultured *B. subtilis* var. *natto* and immunoblotting of the sticky sample. Spots on the white line in the 2D-PAGE of the sticky sample (which is the sticky substance around natto beans) and cultured *B. subtilis* var. *natto* sample (which is used to ferment soybeans to produce natto) indicate proteins commonly detected in the PGA-negative group. The other 30-kDa protein on the white dotted line in the 2D-PAGE of the sticky sample, which yielded a positive reaction in one of the PGA-positive cases. PGA-negative and -positive indicate groups with negative and positive PGA prick test results, respectively. Control, healthy control subjects.

Table 2

Results of skin prick tests for natto, PGA, and nattokinase and ELISA for nattokinase.

Group	No.	Age (years)/sex	Skin prick test						ELISA
			Bean of natto (as is [†])	Sticky substance of natto (as is [†])	Polygamma glutamic acid		Nattokinase		Nattokinase
PGA-negative	1	54/F	2 mm [†]	5 mm [†]	10 mg/mL [‡]	0 mm [†]	0.1 mg/mL [‡]	4 mm [†]	Positive
	2	31/M	4 mm [†]	6 mm [†]	10 mg/mL [‡]	0 mm [†]	not tested		Positive
	3	33/F	6.5 mm [†]	4.5 mm [†]	10 mg/mL [‡]	0 mm [†]	0.1 mg/mL [‡]	3 mm [†]	Positive
	4	20/F	3 mm [†]	3.5 mm [†]	10 mg/mL [‡]	0 mm [†]	0.1 mg/mL [‡]	8.5 mm [†]	Positive
PGA-positive	5 ¹⁶	27/F	5 mm [†]		1 mg/mL [‡]	5.5 mm [†]	not tested		Negative
	6 ¹⁶	41/M	10.5 mm [†]	7.5 mm [†]	0.01 mg/mL [‡]	4 mm [†]	0.1 mg/mL [‡]	0 mm [†]	Negative
	7	21/F	3 mm [†]	3 mm [†]	1 mg/mL [‡]	5.5 mm [†]	0.1 mg/mL [‡]	0 mm [†]	Negative
	8	17/M	0 mm [†]	5.5 mm [†]	10 mg/mL	8.5 mm [†]	0.1 mg/mL [‡]	0 mm [†]	Negative

F, female; M, male; PGA, polygamma glutamic acid.

[†] Mean diameter of a wheal.

[‡] Concentration.

facilities prepare PGA and nattokinase. Thus, additional cases from different parts of Japan should be evaluated in further studies.

The symptoms of PGA-negative/nattokinase-positive cases in the present study were localized to the pharynx with no urticaria. The symptoms suggest that contact urticaria due to nattokinase had occurred; however, the patient reported by Awatani-Yoshidome *et al.* was PGA-negative and nattokinase-positive and experienced anaphylaxis.²⁵ Therefore, the serious symptoms

observed in PGA-sensitized allergies may also occur in nattokinase-sensitized allergies.

PGA-positive cases may be sensitized to PGA during participation in marine sports.⁹ However, only three out of the eight natto allergy cases in this study had a history of engaging in marine sports; therefore, sensitization was likely caused by sources different from those in PGA-negative cases. None of the PGA-positive cases showed atopic dermatitis, whereas all PGA-

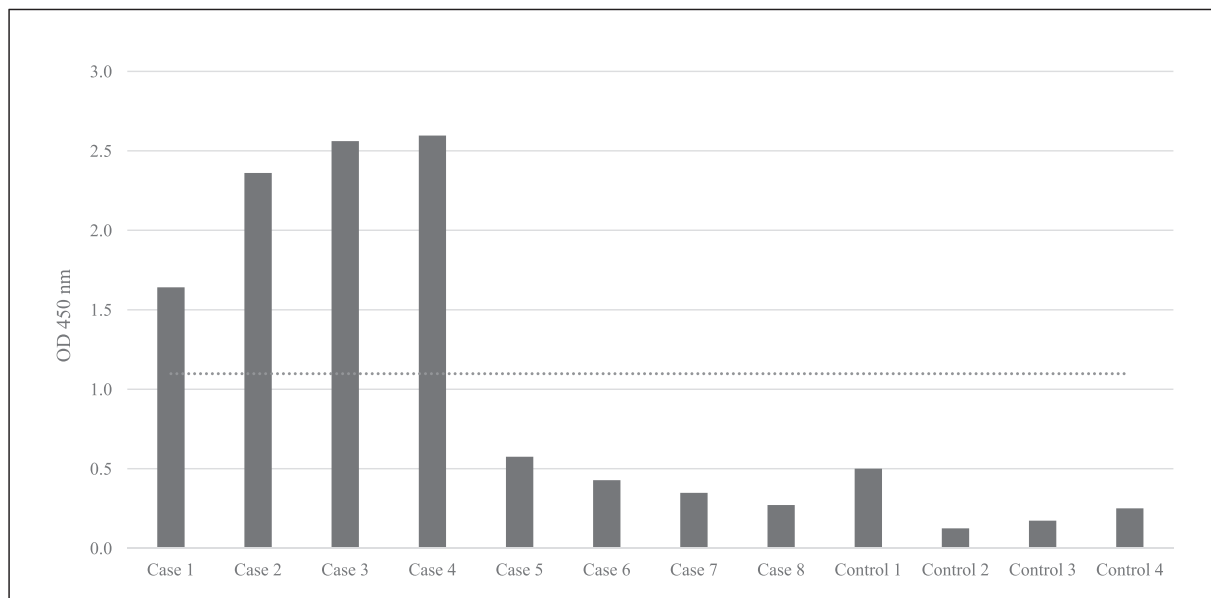


Fig. 3. Results of ELISA for nattokinase. Positivity criteria; average of healthy control subjects +5 SD (dotted line). Control, healthy control subjects.

negative/nattokinase-positive cases presented atopic dermatitis. This result agrees with the findings of Awatani-Yoshidome *et al.*,²⁵ who reported a patient who reacted to nattokinase but not to PGA; in addition, the patient had a history of atopic dermatitis.

The protease activity of a protein reportedly caused barrier dysfunction of the skin and mucosal epithelium, promoted cytokine production, induced a type 2 immune response, and caused sensitization.^{26,27} Thus, sensitization to nattokinase in natto may be induced when the sticky substance of natto attaches around the lips of patients with atopic dermatitis who originally have skin barrier dysfunction. In recent years, subtilisin proteases have been used in laundry detergents,²⁸ and it has been reported that these enzymes induce respiratory type 1 allergy.²⁹ We cannot exclude the possibility that subtilisin proteases in detergents could be primary sensitizers causing the symptoms associated with nattokinase in natto.

Therefore, skin barrier dysfunction in atopic dermatitis may be associated with nattokinase sensitization. We described cases of nattokinase-sensitized natto allergy. Nattokinase is sold as a health supplement that dissolves thrombin by activating fibrinolytic enzymes.^{2,30} Thus, there is a need to instruct nattokinase-sensitized patients to be cautious of not only natto but also supplements. Further studies involving a higher number of patients are needed to confirm our results.

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Conflict of interest

KM is an endowed chair at Hoya Co., Ltd. and received research funding from Hoya. MN and NS are employees of Hoya. The rest of the authors have no conflict of interest.

KS wrote the initial draft of the manuscript. KS and KF performed the skin prick tests. NS and MN performed ELISA and immunoblotting, respectively. KM and AY assisted with the study and preparation of the manuscript. All authors have read and approved the final manuscript.

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