

Ani s 1, the major allergen of *Anisakis simplex*: Purification by affinity chromatography and functional expression in *Escherichia coli*

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Abstract

Third stage larvae of the nematode *Anisakis simplex* often infect marine fish and invertebrates. When the larvae are ingested orally via seafood, they can cause IgE-mediated allergic reactions as well as anisakiasis. Of the known *A. simplex* allergens, Ani s 1 (Kunitz/bovine pancreatic trypsin inhibitor family protein) has been demonstrated to be a major allergen, being expected to be a useful tool for diagnosis of *A. simplex* allergy. For a diagnostic purpose, sufficient amounts of either natural Ani s 1 (nAni s 1) or recombinant Ani s 1 (rAni s 1) with an IgE-binding capacity should be stably supplied whenever needed. In this study, therefore, we first developed a simple and rapid purification method for Ani s 1 that is based on affinity chromatography using anti-Ani s 1 antibodies as ligands. The method was shown to produce nAni s 1 with a higher yield than the previously reported methods. Then, an attempt was made to express rAni s 1 in *Escherichia coli* as a His-tagged protein. rAni s 1 obtained as an inclusion body was solubilized in a solvent containing denaturing and reducing reagents and purified by nickel-chelate chromatography. Refolding of rAni s 1 was accomplished by dialysis in the presence of arginine, followed by that in the absence of arginine. Fluorescence ELISA and inhibition ELISA data revealed that rAni s 1 is IgE reactive enough to be used as a diagnostic tool.

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1. Introduction

Third stage larvae of the nematode *Anisakis simplex* often infect marine fish and invertebrates. When the larvae are ingested orally via seafood, they occasionally penetrate the human gastrointestinal mucosa and cause a disease called anisakiasis. Typical symptoms of anisakiasis are nausea, diarrhea, abdominal pain and vomiting [1]. Moreover, it is known that infestation with *A. simplex* makes the Th2 subset active in immune systems of certain individuals, leading to the generation of high amounts of immunoglobulin E (IgE) antibodies [2]. In subsequent infection

by the nematode, therefore, those individuals can develop IgE-mediated allergic reactions ranging from urticaria to life-threatening anaphylactic shock [3–7].

Of the eight *A. simplex* allergens so far identified [8–15], Ani s 1 (Kunitz/bovine pancreatic trypsin inhibitor family protein) has been demonstrated to be a major allergen. It is recognized by more than 80% of *Anisakis*-allergic patients and hence is expected to be a useful tool for diagnosis of *A. simplex* allergy [16,17]. To utilize Ani s 1 as a diagnostic tool, sufficient amounts of either natural Ani s 1 (nAni s 1) or recombinant Ani s 1 (rAni s 1) with an IgE-binding capacity should be stably supplied whenever needed. At present, two purification methods are available for nAni s 1. However, both methods are complicated and time-consuming, resulting in a very low yield of nAni s 1 (for example, only a yield of 2.2 µg from 1 g of *A. simplex* in our method [9]). To overcome this problem, a sophisticated purification method for Ani s 1 should be devised. As for rAni s 1, Rodriguez-Perez et al. [18] have recently expressed Ani s 1 in the yeast *Pichia pastoris*. Although only

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; IPTG, isopropyl-β-D-thiogalactoside; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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qualitatively, the IgE-binding capacity of rAni s 1 has been shown to be comparable to that of nAni s 1 by inhibition immunoblotting. However, the yield of rAni s 1 (0.8 mg from 1 l of the yeast culture) may be still insufficient for the routine diagnosis of *A. simplex* allergy. In relation to this, it should be noted that the *Escherichia coli* expression system is generally known to produce much higher amounts of recombinant proteins than the yeast expression system. In our previous attempt to express Ani s 1 in *E. coli*, however, rAni s 1 was obtained only in an insoluble form (i.e., as an inclusion body), probably due to the incorrect folding [9]. For a diagnostic purpose, it is necessary to produce soluble rAni s 1 by refolding.

In this paper, we report the development of a rapid and efficient purification method for nAni s 1 by affinity chromatography using anti-Ani s 1 antibodies as ligands and the production of refolded rAni s 1 that is not only soluble in aqueous solvents but also IgE reactive.

2. Materials and methods

2.1. Production of antiserum to Ani s 1

Antiserum to Ani s 1 was raised in a 11-week-old female Japanese white rabbit (JW/CSK; Sankyo Labo Service, Tokyo, Japan), using rAni s 1 (refer to the Section 2.4 for the production of rAni s 1) as the antigen. The rabbit received both subcutaneous (1.4 ml) and intramuscular (0.6 ml) injections of rAni s 1 suspension (1.76 mg/ml) in Freund's complete adjuvant. Four and 10 weeks later, the rabbit was intravenously injected via ear vein with 0.5 ml of rAni s 1 solution (3.52 mg/ml) without adjuvant. Blood (about 20 ml) was taken from the ear vein 6 times every two weeks starting from the 5th week after the primary immunization, kept at 4 °C overnight and centrifuged to obtain serum. All sera were pooled and used as an anti-Ani s 1 serum.

2.2. Preparation of an anti-Ani s 1 antibody column

One milliliter of rProtein A Sepharose Fast Flow beads (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was washed well with phosphate buffered saline (PBS; 150 mM NaCl in 10 mM phosphate buffer, pH 7.0) and incubated with 5 ml of the antiserum and 4 ml of PBS at room temperature for 1 h. After being washed with 200 mM sodium borate–HCl buffer (pH 9.0), the beads were added with 10 ml of the same buffer containing 20 mM dimethyl pimelimidate as a cross-linker and incubated at room temperature for 30 min. Then, the beads were washed twice with 10 ml of 200 mM ethanolamine–HCl buffer (pH 8.0) and incubated with 10 ml of the same buffer at room temperature for 2 h to arrest the cross-linking reaction. Finally, the beads coupled with rabbit IgG were washed well with PBS and packed in a column (0.5 × 3.8 cm), which is called “anti-Ani s 1 antibody column” in this paper.

2.3. Purification of nAni s 1

Third stage larvae of *A. simplex*, which were collected from the surface of hepatopancreas of walleye pollack (*Theragra*

chalcogramma), were homogenized with two volumes of PBS containing a protease inhibitor cocktail (Sigma-Aldrich Japan, Tokyo, Japan) and centrifuged at 18,800 *g* for 15 min. The precipitate was similarly extracted once more with PBS. The supernatants (crude extract) were combined and applied to affinity chromatography using the anti-Ani s 1 antibody column. After being washed with 10 ml of PBS to remove unadsorbed proteins, the column was eluted with 1 ml of 100 mM glycine–HCl buffer (pH 2.0). The eluate was immediately added with 100 µl of 1 M Tris–HCl buffer (pH 8.0) for neutralization and then subjected to reverse-phase HPLC on a TSKgel Phenyl-5PW RP column (0.46 × 7.5 cm; Tosoh, Tokyo, Japan). Elution of the column was achieved by a linear gradient of acetonitrile (0–42% in 20 min) in 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min. Proteins were monitored at 220 nm with a UV detector. The eluate corresponding to Ani s 1 was manually collected and lyophilized. In the subsequent experiments, the dried product was dissolved in PBS and used as an nAni s 1 solution.

2.4. Expression, purification and refolding of rAni s 1

In this study, rAni s 1 was expressed in *E. coli* as a His-tagged protein. A cDNA coding for mature Ani s 1 was amplified by polymerase chain reaction (PCR), using the previously purified plasmids [9] as a template. The following primers were used for PCR: a forward primer with *StuI* restriction site (underlined) 5'-ATAGGCCTGATAGAACGGAATGTC-3' and a reverse primer with *HindIII* restriction site (underlined) 5'-GGAAGCTTTTATTTACAACAATTTGCAAATAAT-3'. Amplification was performed using HotMaster Taq DNA Polymerase (Eppendorf, Hamburg, Germany) under the following conditions: 94 °C for 2 min and 35 cycles of 94 °C for 20 s, 55 °C for 10 s and 70 °C for 50 s. The PCR product and the expression vector pQE-30 Xa (Qiagen, Hilden, Germany) were digested with *StuI* and *HindIII* and ligated using a DNA Ligation Kit (Takara, Otsu, Japan). *E. coli* JM109 was transformed with the ligation product and cultured on LB agar containing 0.005% ampicillin at 37 °C overnight. A single colony, for which the correct insertion of the Ani s 1 cDNA into the expression vector was confirmed by DNA sequencing, was selected and cultured in 500 ml of LB medium containing 0.005% ampicillin at 37 °C until the absorbance at 600 nm reached 0.7. To induce protein expression, the medium was added with isopropyl-β-D-thiogalactoside (IPTG) at a concentration of 1 mM and further incubated for 3 h. Bacteria were harvested by centrifugation, suspended in 25 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl and 1 mM EDTA and sonicated well. After centrifugation, the precipitate containing the His-tagged rAni s 1 was dissolved in 40 ml of 20 mM phosphate buffer (pH 7.4) containing 500 mM NaCl, 10 mM imidazole, 6 M guanidine hydrochloride and 5 mM 2-mercaptoethanol by sonication. Purification of His-tagged rAni s 1 was performed by nickel-chelate chromatography on a HisTrap Chelating HP column (GE Healthcare Bio-Sciences) as recommended by the manufacturer. Refolding of rAni s 1 was achieved by dialysis against 20 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, 1 mM CaCl₂ and 400 mM L-arginine,

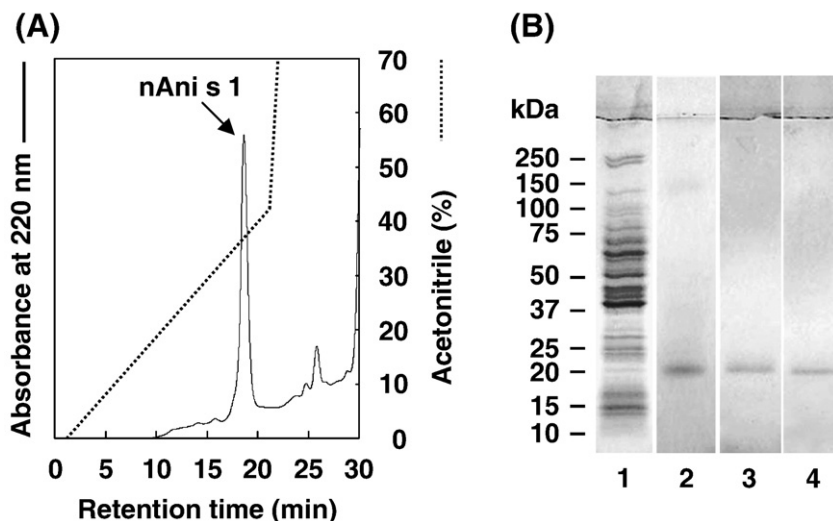


Fig. 1. Purification of nAni s 1. (A) Reverse-phase HPLC. Sample: adsorbed fraction obtained by affinity chromatography. (B) SDS-PAGE. Lanes: 1, crude extract from *A. simplex*; 2, partially purified Ani s 1 after affinity chromatography; 3 and 4, purified Ani s 1 after reverse-phase HPLC. Reduced and non-reduced conditions were employed for lanes 1–3 and 4, respectively.

followed by dialysis against the same solvent devoid of L-arginine. Finally, the inner solution was centrifuged and the supernatant was used as the refolded rAni s 1 solution.

2.5. Human sera

Sera were donated from 10 patients with clinical histories of allergic reactions, such as urticaria and anaphylaxis, after eating raw or cooked fish. Informed consent was obtained from each patient. All patients had been diagnosed to be allergic to *A. simplex* but not to fish at hospital, based on the determined CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) classes of 2–6 against *A. simplex* and 0 against fish such as mackerel and tuna. As described below, our fluorescence ELISA also showed that all patients have specific IgE to Ani s 1. Serum samples were stored at -20°C until use.

2.6. Enzyme-linked immunosorbent assay (ELISA)

IgE reactivity of either nAni s 1 or rAni s 1 was evaluated by fluorescence ELISA, which was performed as reported previously [19]. In brief, various concentrations (1–300 nM) of antigen solution were coated on a 96-well microtiter plate and reacted successively with patient serum (diluted 1:100) and β -galactosidase-conjugated goat anti-human IgE antibody (0.25 $\mu\text{g}/\text{ml}$; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out using 4-methylumbelliferyl- β -D-galactoside as a substrate. Fluorescence units were determined on a SPECTRAMax GEMINI XS (Molecular Devices, Tokyo, Japan) with excitation and emission wavelengths at 367 and 453 nm, respectively. For inhibition ELISA, each patient serum (diluted 1:50) was preincubated with an equal volume of 2000 nM nAni s 1 or rAni s 1 solution at 37°C for 2 h and added to a microtiter plate that had previously been coated with 30 nM nAni s 1 solution. The subsequent procedure was the same as

described for ELISA. All ELISAs (including inhibition ELISA) were performed in triplicate and the data were expressed in mean $\pm$ SD.

2.7. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a PhastSystem apparatus (GE Healthcare Bio-Sciences) according to the manufacturer's instructions. Each sample was dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing only 2% SDS or containing 2% SDS, 100 mM dithiothreitol and 6 M urea, heated at 70°C for 10 min and run on a PhastGel Gradient 8–25 gel (GE Healthcare Bio-Sciences). Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as references. Proteins were detected by staining with Coomassie Brilliant Blue R-250.

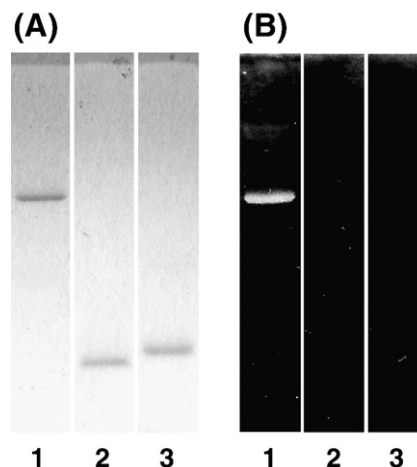


Fig. 2. Detection of carbohydrates. Lanes: 1, transferrin; 2, purified nAni s 1; 3, purified rAni s 1. (A) SDS-PAGE under a reduced condition. (B) Carbohydrate staining using the ECL Glycoprotein Detection Module.

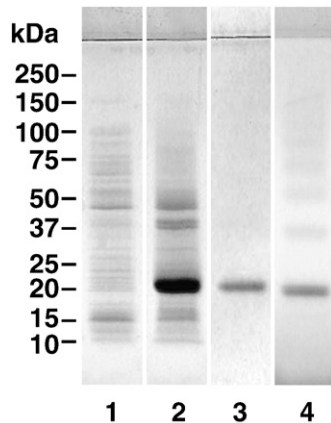


Fig. 3. Analysis of expression and purification of rAni s 1 by SDS-PAGE. Lanes: 1, soluble fraction from IPTG-induced *E. coli*; 2, insoluble fraction from IPTG-induced *E. coli*; 3 and 4, purified rAni s 1. Reduced and non-reduced conditions were employed for lanes 1–3 and 4, respectively.

2.8. Chemical analysis

Protein was determined with a Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories), using bovine serum albumin as a standard. The presence or absence of carbohydrate moieties in nAni s 1 and rAni s 1 was examined using an ECL Glycoprotein Detection Module (GE Healthcare Bio-Sciences) as recommended by the manufacturer. Amino acid sequencing was performed with an automatic gas-phase protein sequencer (LF-3400D TriCart with high sensitivity chemistry; Beckman Coulter, Fullerton, CA, USA).

3. Results and discussion

3.1. Purification of nAni s 1

nAni s 1 was easily purified by affinity chromatography using the anti-Ani s 1 antibody column, followed by reverse-

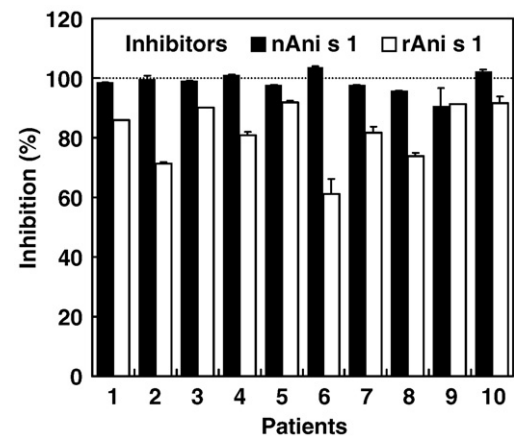


Fig. 5. Inhibition ELISA. Inhibitory ability of nAni s 1 and rAni s 1 against the reactivity of patient sera to nAni s 1 was evaluated by fluorescence ELISA. Each datum is expressed in mean $\pm$ SD ($n=3$).

phase HPLC (Fig. 1). When analyzed by SDS-PAGE, Ani s 1 with a molecular mass of 19 kDa was not clearly recognized in the crude extract from *A. simplex* (lane 1 in Fig. 1B). In affinity chromatography, the majority of proteins contained in the crude extract passed through the anti-Ani s 1 antibody column, while Ani s 1, together with small amounts of impurities (about 150 kDa), was recovered in the adsorbed fraction (lane 2 in Fig. 1B). Following removal of impurities in the adsorbed fraction by reverse-phase HPLC (Fig. 1A), highly pure nAni s 1 was obtained (lanes 3 and 4 in Fig. 1B). Analysis by a protein sequencer identified N-terminal 10 amino acid residues of purified nAni s 1 as follows: DRTECQLPLD. This sequence is completely identical with that reported in our previous paper [9]. The purification method developed in this study is much simpler and more time-saving than our previous method consisting of four chromatographic steps [9]. More importantly, the yield of nAni s 1 (40 μ g/g of larvae) was greatly improved compared to the previous value (200 μ g/90 g = 2.2 μ g/g) [9].

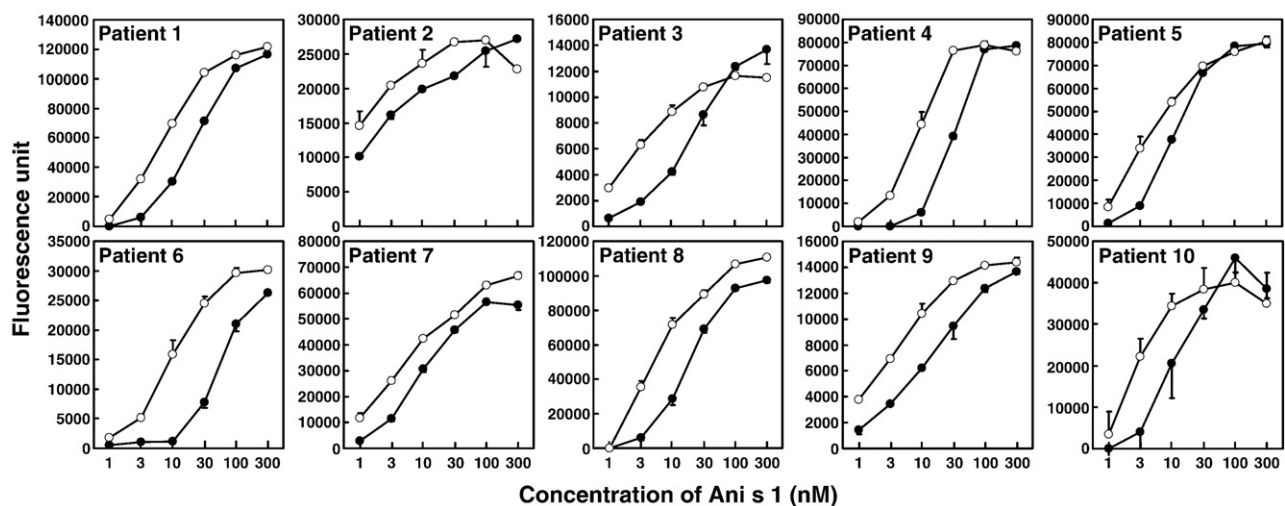


Fig. 4. Analysis of the reactivity of patient sera to nAni s 1 (open circle) and rAni s 1 (closed circle) by fluorescence ELISA. Each datum is expressed in mean $\pm$ SD ($n=3$).

In experiments using the ECL Glycoprotein Detection Module, transferrin used as a glycoprotein reference was positive to carbohydrate staining, while no carbohydrate moieties were detected in the purified nAni s 1 (Fig. 2). Rodríguez-Pérez et al. [18] recently reported that rAni s 1 expressed in the yeast system is positive to carbohydrate staining. However, their mass spectrometric analysis suggested that not all but only a part of the recombinant molecules are glycosylated. In view of our finding that nAni s 1 is not a glycoprotein, it is assumed that erroneous glycosylation have occurred during expression of rAni s 1 in the yeast system.

3.2. Expression, purification and refolding of rAni s 1

In our previous study, rAni s 1 expressed in *E. coli* as a glutathione-*S*-transferase-fusion protein was obtained as an inclusion body in the insoluble fraction from IPTG-induced bacteria [9]. An attempt was made to express rAni s 1 in *E. coli* as a His-tagged protein in this study but the results were essentially the same as in our previous study. His-tagged Ani s 1 was not recovered in the soluble fraction of IPTG-induced bacteria but in the insoluble fraction as an inclusion body (lanes 1 and 2 in Fig. 3). In SDS-PAGE, purified nAni s 1 migrated at a position of 19 kDa under both non-reducing and reducing conditions (lanes 3 and 4 in Fig. 1), suggesting that 17 Cys residues contained in Ani s 1 are associated with intrachain disulfide bridges but not with interchain disulfide bridges. However, it is very likely that recombinant molecules expressed in *E. coli* formed interchain disulfide bridges leading to aggregation and eventual precipitation. In this study, insoluble rAni s 1 was first dissolved in the solvent containing both denaturing (guanidine hydrochloride) and reducing (2-mercaptoethanol) reagents and purified by nickel-chelate chromatography. In the next step, refolding of rAni s 1 was performed by dialysis in the presence of L-arginine, which is known to be able to suppress aggregation of proteins during a refolding process [20,21]. As analyzed by SDS-PAGE under a reduced condition, refolded rAni s 1 migrated slightly slower than nAni s 1 because of the addition of His-tag to the N-terminus, affording a single band of 20 kDa (lane 3 in Fig. 3). Under a non-reduced condition, however, refolded rAni s 1 produced ladder bands corresponding to multimers as well as a major band of 20 kDa (lane 4 in Fig. 3). These results suggested that, due to the partial incorrect refolding, small amounts of multimers linked by interchain disulfide bridges are still contained in the final preparation of rAni s 1. In a typical run, the yield of rAni s 1 was as much as 24.6 mg/l culture medium, which was 30-fold higher than that (0.8 mg/l) previously obtained in the yeast expression system [18]. Since the *E. coli* expression system was employed in this study, no glycosylation was naturally recognized with the rAni s 1 preparation (lane 3 in Fig. 2B).

3.3. IgE reactivity of nAni s 1 and rAni s 1

As analyzed by fluorescence ELISA using 10 patient sera, both nAni s 1 and rAni s 1 exhibited IgE reactivity in a concentration-dependent manner (Fig. 4). On the whole, however, patient sera,

although rather small in number, reacted more strongly to nAni s 1 than to rAni s 1. In consistency with this, inhibition ELISA experiments showed that the reactivity of patient sera to nAni s 1 was completely or almost completely inhibited ($99 \pm 4\%$ inhibition) by preincubation with nAni s 1 but not completely ($82 \pm 10\%$ inhibition) by that with rAni s 1 (Fig. 5). Based on these results, the IgE-binding capacity of rAni s 1 was judged to be somewhat lower than that of nAni s 1. As described above, incorrectly refolded molecules forming multimers are suggested to be included in the rAni s 1 preparation, accounting for the lower IgE-binding capacity of rAni s 1 than nAni s 1.

In this study, we developed a simple and rapid purification method for nAni s 1 that is based on affinity chromatography using anti-Ani s 1 antibodies as ligands. The method can produce nAni s 1 with a higher yield than the previously reported method [9]. Although the use of nAni s 1 is desirable for accurate diagnosis of *A. simplex*, it may be difficult to secure sufficient amounts of third stage larvae for the purification of nAni s 1. Therefore, rAni s 1 with an IgE-binding capacity was expressed in *E. coli* as an alternative of nAni s 1. rAni s 1 was not only abundantly yielded but also satisfactorily IgE reactive although somewhat less potently than nAni s 1. Conclusively, our rAni s 1 preparation expressed in the *E. coli* system will be a useful tool for future diagnosis of *A. simplex* allergy.

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