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Letter to the Editor

Identification and gene cloning of a new major allergen Cha o 3 from *Chamaecyparis obtusa* (Japanese cypress) pollen

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29 **Clinical implications**

30 Data regarding Cha o 3 may lead to the development of new ASIT strategies to control

31 allergic symptoms throughout the spring allergy season and enhance understanding of

32 the clinical pathology of Cupressaceae pollinosis.

33

34 **Capsule summary**

35 We discovered a novel major allergen, Cha o 3, from Japanese cypress pollen, a known

36 cause of common pollinosis. Our findings may lead to development of new allergen

37 immunotherapies and enhance understanding of Cupressaceae pollinosis.

38

39 **Key words**

40 Allergen, *Chamaecyparis obtusa*, Japanese cypress, pollinosis, Cha o 1, Cha o 2, Cha o

41 3, allergen immunotherapy, pollen, Cupressaceae

42

43 **Abbreviations used**

44 cDNA: complementary DNA

45 ASIT: allergen-specific immunotherapy

46 BAT: basophil activation test

47 PAS: periodic acid-Schiff

48 rCha o 2: recombinant Cha o 2

49 SBP: Sugi (Japanese cedar) basic protein
50 RACE: rapid amplification of cDNA ends
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73 To the Editor:

74 Trees of the Cupressaceae family produce many types of pollen. As typified by
75 mountain cedar, these pollens cause pollinosis worldwide.[1] In Japan, the Cupressaceae
76 family member *Chamaecyparis obtusa* (Japanese cypress) has been re-planted over a
77 substantial area.[2] Japanese cypress pollinosis is one of the most common allergic
78 diseases, and the increasing number of patients with Japanese cypress pollinosis will
79 remain a concern in the near future.

80 Cha o 1 and Cha o 2 are major allergens of Japanese cypress pollen, and they exhibit
81 considerable amino acid sequence homology with the major Japanese cedar pollen
82 allergens Cry j 1 and Cry j 2, respectively.[3, 4] Consistent with this sequence homology,
83 cross-reactivity with some T cell epitopes and IgE has been demonstrated.[5, 6] Due to
84 their cross-reactivity, both Cry j– and Cha o 1–specific Th2 responses are reportedly
85 inhibited in patients who have received allergen-specific immunotherapy (ASIT) using
86 standardized Japanese cedar pollen extract.[2] However, allergic symptoms and QOL may
87 worsen despite ASIT during Japanese cypress season, suggesting that unidentified
88 allergens unique to Japanese cypress pollen exist.[2] In this study, we explored the third
89 major allergen to be isolated from Japanese cypress pollen extract after Cha o 1 and Cha
90 o 2.

91 On SDS-PAGE analysis of a crude extract of cypress pollen, we observed a diffuse
92 major band (Cha o 3) at approximately 63 kDa (Fig. 1A). Cha o 3 was isolated using ion
93 exchange chromatography (see the Methods section in this article's Online Repository
94 at www.jacionline.org). Cha o 3 exhibited double bands (63 and 66 kDa) under both
95 non-reducing and reducing conditions (Fig. 1A and 1B). The Cha o 3 double bands were
96 PAS-stain positive, and the same peptide fragments were detected by LC-MS/MS from

97 digests of each band (data not shown). Therefore, we concluded that the Cha o 3 double
98 bands were the result of glycosylation difference, as with Cha o 1.[3]
99 To confirm whether Cha o 3 is an allergen in humans, an ELISA was performed to
100 detect Cha o 3–specific IgE in the serum of 16 Japanese cypress pollinosis patients. In
101 addition to Cha o 1–specific IgE, a high frequency (14 of 16 patients, 87.5%) of Cha o
102 3–specific IgE binding was observed (see Table E1 in this article's Online Repository at
103 www.jacionline.org). Surprisingly, a more significant correlation was observed between
104 Cha o 3–specific IgE and Japanese cypress–specific IgE quantified by ImmunoCAP
105 than Cha o 1 (Fig. 1D and 1E), suggesting that Cha o 3 is a previously undescribed
106 Japanese cypress pollen allergen in humans that contributes significantly to diagnostic
107 scores. In addition, BAT results indicated that Cha o 3–specific IgE is functional in
108 patients (see Fig. E1 in this article's Online Repository at www.jacionline.org). A Cha o
109 3–dominant response was observed in patients with Japanese cypress pollinosis (Fig.
110 E1C), suggesting that Cha o 3 is involved in the pathogenic mechanism of Japanese
111 cypress pollinosis along with Cha o 1.
112 A lymphocyte stimulation test was used to elucidate the details of the pathogenesis of
113 Cha o 3. PBMCs obtained from 16 patients showed a proliferative response to Cha o 3
114 of the same or greater magnitude than other cypress allergens (Fig. 2). Furthermore, the
115 ratio of Cha o 3–specific T cells among proliferating lymphocytes was higher than that
116 observed with other Japanese cypress allergens.
117 Molecular cloning of the Cha o 3 gene was also carried out (see the Methods section in
118 this article's Online Repository at www.jacionline.org). The complete deduced amino
119 acid sequence of the gene product comprised 556 residues, with a calculated molecular
120 weight of 61,636 Da (see Fig. E2 in this article's Online Repository at

www.jacionline.org). The sequence from Met^{1} to Ser^{28} likely represents a signal peptide based on the in silico prediction. In addition, completely consistent results were observed by N-terminal amino acid sequence analysis (see Fig. E2). Therefore, the estimated molecular weight of mature Cha o 3 is 58,865 Da. Cha o 3 did not exhibit any sequence identity with known Cry j or other Cha o allergens. A Pfam-A search indicated that Cha o 3 contains a cellulase (glycosyl hydrolase family 5) motif from Pro^{70} to Gly^{353} (see Fig. E3A in this article's Online Repository at www.jacionline.org). The deduced cellulase motif of Cha o 3 was highly conserved compared with other identified plant cellulases (see Fig. E3B). As cellulolytic activity was detected in our preliminary study (data not shown), we concluded that Cha o 3 is a cellulase. As the anthers of sweet pea reportedly contain large amounts of cellulase associated with pollen maturation,[7] high expression of Cha o 3 in cypress pollen is reasonable. Our findings, therefore, suggest that proteins in the cellulase family may be novel allergens in the pollen of other plants, particularly trees of the Cupressaceae family.

Finally, in order to confirm the cross-reactivity of Cha o 3 with Japanese cedar allergen, we evaluated the production of IL-5, a typical Th2 cytokine, in PBMCs isolated from another panel of Japanese cedar pollinosis patients, with and without Japanese cedar–specific immunotherapy. As expected, Cry j 1–, Cry j 2–, and Cha o 1–associated IL-5 production was significantly suppressed in patients who received ASIT (see Fig. E4 in this article's Online Repository at www.jacionline.org; 96.0, 97.5, and 92.5% inhibition compared with ASIT-naïve patients, respectively). Interestingly, Cha o 3–associated IL-5 production was also inhibited in patients who received ASIT (67.5% inhibition). However, Cha o 3 induced the highest level of IL-5 secretion among the five allergens in patients who received ASIT, suggesting that ASIT using Japanese cedar

145 extract might not completely control Cha o 3–specific Th2 responses.
146 These data raise two important points. First, it is unlikely that Cha o 3 is a unique
147 allergen in Japanese cypress pollen due to the deduced presence of Cha o 3 homologs
148 that are predicted to cross-react with Cha o 3 in Japanese cedar pollen, similar to the
149 relationship between Cry j 1 and Cha o 1. Second, the levels of Cha o 3 homologs in
150 Japanese cedar pollen extract may not be sufficient for desensitization, or their
151 homology may be low. It is noteworthy that Cupressaceae pollens share allergenic
152 components. For example, Cha o 1 has high (>85%) sequence identity to Jun a 1 (from
153 mountain cedar) and Jun v 1 (from eastern red cedar).[8, 9] Further experiments aimed at
154 identifying Cha o 3 homologs in cedar pollen are needed to enhance understanding of
155 Japanese cedar and cypress pollinosis.
156 In conclusion, we demonstrated that Cha o 3 is a novel major allergen of Japanese
157 cypress pollen. The allergen was designated Cha o 3 according to WHO/IUIS Allergen
158 Nomenclature Subcommittee recommendations. New ASIT approaches using Cha o 3 in
159 addition to standardized Japanese cedar extract may be useful for improving the efficacy
160 of ASIT throughout the cypress pollen season. Moreover, the present results suggest the
161 presence of a previously undescribed allergen in Japanese cedar pollen. We hope the
162 discovery of Cha o 3 will contribute to the development of basic research and
163 approaches for treating Japanese cedar, cypress, and Cupressaceae pollinosis.
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216

217 **Figure legends**

218 **Figure 1**

219 SDS-PAGE analysis and correlation between ImmunoCAP values and Cha o
220 allergen–specific IgE. (A) Cha o 3: purified Cha o 3 under non-reducing conditions,
221 Cha o 1: purified Cha o 1 under non-reducing conditions; (B) purified Cha o 3 under
222 reducing conditions, (C) purified recombinant Cha o 2. Because only a limited amount
223 of Cha o 2 was obtained from the cypress extract, recombinant Cha o 2 was prepared.
224 (D) Correlation between ImmunoCAP values and Cha o 3–specific IgE binding
225 measured by ELISA or (E) Cha o 1–specific IgE. Statistical analysis was performed
226 using the Pearson's correlation coefficient test. Lines indicate 95% probability ellipses.
227

228 **Figure 2**

229 Proliferative response of PBMCs. PBMCs were stimulated with antigen (10 μg/mL) for
230 3 days. After incubation for an additional 16 hours with [^{3}H]-thymidine, [^{3}H]-thymidine
231 incorporation was measured. Statistical significance versus Cha o 3 was evaluated using
232 Dunnett's test (*$p < 0.01$).
233

234 **Footnote**

235 The cDNA sequence of Cha o 3 was deposited in the GenBank sequence database under
236 accession number HV942721.

1 **Online Repository text**

2 **Figure legends**

3 **Figure E1**

4 Cha o 3 induces basophil activation in Japanese cypress pollinosis patients. (A) Whole
5 blood of V043 was stimulated with antigen (0.1 μg/mL) or anti–human IgE (10 μg/mL)
6 as a positive control for 15 minutes at 37°C. Whole blood of (B) V016 or (C) V037 was
7 stimulated with each allergen (1 ng/mL).

8

9 **Figure E2**

10 Sequence of cloned cDNA of Cha o 3 and deduced amino acid sequence. Domains to
11 which primers were applied for PCR cloning are showed by arrows (black arrows: first
12 primers, blue arrows: second primers, red arrows: primers for 5'-RACE, green arrow:
13 primer for 3'-RACE). Arrow direction indicates forward or reverse primers. The blue
14 box represents the predicted signal peptide. Peptides which were identified by amino
15 acid sequence analysis are shown in red boxes. Several independently isolated cDNA
16 clones identified polymorphisms at nucleotides 88 (C or A), 264 (A or C), 681 (C or T),
17 772 (G or A), 792 (A or G), 870 (C or T), 895 (G or A), and 1029 (G or A). Nearly half
18 of the mutations encoded the same amino acids, except nucleotides 88 (Pro or Thr), 772
19 (Val or Ile), and 895 (Val or Ile). Due to these polymorphisms, the most frequent cDNA
20 clone is discussed in this report.

21

22 **Figure E3**

23 (A) Predicted Cha o 3 protein. (B) ClustalW multiple sequence alignment for homology
24 analysis. The predicted cellulase domain of Cha o 3 (from Pro^{70} to Gly^{353}) was

25 compared with the cellulases of *Medicago truncatula* (accession number KEH20742),
26 *Theobroma cacao* (accession number XP_007044872), and *Arabidopsis thaliana*
27 (accession number NP_172772). Shaded areas indicate consistent amino acid sequence
28 compared with Cha o 3.
29
30 **Figure E4**
31 IL-5 production by PBMCs collected from Japanese cedar and cypress pollinosis
32 patients with or without Japanese cedar–specific immunotherapy. PBMCs were
33 stimulated with 10 μg/mL of the indicated allergens for 72 hours. The IL-5 content of
34 the supernatant was determined by ELISA. Statistical analyses were performed using
35 the two-tailed Student's t test (*$p < 0.01$).
36
37 **Table E1**
38 Levels of allergen-specific IgE antibody in patient serum samples. Allergen-specific IgE
39 binding was determined by ELISA. IgE binding against Cha o 1 or Cha o 3 was
40 determined to be positive when the OD_{450} value was more than twice that of negative
41 control serum. Japanese cedar and cypress pollen–specific IgE were evaluated using the
42 ImmunoCAP method.
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49 **Methods**

50 **Plant materials**

51 Pollen from Japanese cypress was purchased from the Japan Forest Tree Breeding
52 Association (Tokyo, Japan). Female flowers were collected from Japanese cypress trees
53 located in Hanno-shi Saitama, Japan, immediately before the pollen season. Pollen and
54 female flowers were stored at −80°C prior to use.

55

56 **Subjects**

57 Patients were selected from the employees of Hanno Research Center, Taiho
58 Pharmaceutical Co., Ltd., on the bases of clinical symptoms of seasonal allergic rhinitis
59 suffered every spring. All serum samples were measured for Japanese cedar pollen and
60 Japanese cypress pollen–specific IgE using ImmunoCAP (at the clinical test service of
61 SRL, Inc., Tokyo, Japan). Finally, we enrolled 16 patients who were determined to be
62 specific-IgE seropositive (at least ImmunoCAP class 2, ≥0.71 U_A/mL) to both kinds of
63 pollen. All studies were approved by the Institutional Ethical Review Board for basic
64 research. For the determination of allergen-specific IL-5 production by PBMCs, 47
65 patients who were diagnosed with Japanese cedar and Japanese cypress pollinosis by
66 the Department of Otolaryngology-Head & Neck Surgery, Okayama University
67 Hospital, were enrolled in this study after providing informed consent. A total of 24 of
68 47 patients received subcutaneous allergen-specific immunotherapy (ASIT) using a
69 standardized extract of Japanese cedar pollen (Torii Pharmaceutical Co., Ltd., Tokyo,
70 Japan) over a period of at least 2 years, as described in a previous report.[E1]

71

72 **Isolation of native Cha o 1 and Cha o 3 protein from Japanese cypress pollen**

Approximately 5 g of Japanese cypress pollen was homogenized in 25 mL of neutral buffer (10 mM Tris-HCl, pH 7.8) using a Teflon homogenizer. After incubation for 1 hour at 4°C, this crude extract was centrifuged at 12,000 × *g* for 20 minutes at 4°C. The supernatant was then subjected to Vivapure Q anion exchange chromatography (Sartorius Stedim, Göttingen, Germany), and the flow-through was collected. This unadsorbed fraction was dialyzed against 10 mM acetate buffer (pH 5.0) and applied to a Vivapure S cation exchange column (Sartorius Stedim) equilibrated in the same 10 mM acetate buffer (pH 5.0) to adsorb Cha o 3 onto the carrier resin. Adsorbed Cha o 3 was eluted by gradient elution using NaCl solution in the neutral buffer described above. Cha o 3 was eluted in neutral buffer containing 0.5 M NaCl. Cha o 1 was isolated from crude extract of Japanese cypress pollen using a previously reported method.[E2] Contamination of LPS in the purified Cha o 1 and Cha o 3 samples was examined by Limulus Amebocyte lysate Pyrosta ES-F single test (Wako Pure chemical, Osaka, Japan. Detection limit is 0.015 EU/mL) and the levels were respectively not detectable and <1 EU/mL.

Expression of recombinant Cha o 2 protein

Because the amount of Cha o 2 from cypress extract was not sufficient to use in the various experiments, recombinant Cha o 2 was prepared according to a previous report.[E3,E4] Briefly, the Cha o 2 cDNA fragment was synthesized and subcloned into pENTR4 vector (Life Technologies, MA, USA) through *Bam*HI and *Xho*I sites. The subcloned Cha o 2 cDNA was recombined into pEXP1-DEST expression vector using Gateway® LR Clonase® enzyme mix. The resulting Cha o 2 expression vector was transformed into *E. coli* (BL21 STAR, Life Technologies) for protein expression.

97 Expression of rCha o 2 was induced in 0.6 mM isopropyl-β-D-1-thiogalactopyranoside
98 for 6 hours. After the lysis of *E. coli* cells, rCha o 2 in the lysate was purified using Ni^{2+}
99 NTA resin. The purity and molecular weight of rCha o 2 was confirmed by SDS-PAGE.
100 The presumed molecular weight of rCha o 2 was approximately 59 kDa because of the
101 inclusion of 6×His, Xpress® tag, and spacer peptides in the pEXP1-DEST vector in
102 addition to rCha o 2. Contamination of LPS in the rCha o 2 sample was not detected.
103
104 **Characterization of purified Cha o 3 protein**
105 Purified Cha o 3 was analyzed by SDS-PAGE using a Novex® NuPAGE® SDS-PAGE
106 system (Life Technologies) and a general protocol. Gel staining was performed using
107 SYPRO® Ruby Protein Gel Stain (Lonza, Basel, Switzerland), and protein bands were
108 detected using ImageQuant LAS 4010 (GE Healthcare, Little Chalfont, UK). N-terminal
109 and internal amino acid sequence analysis of purified Cha o 3 was conducted by Toray
110 Research Center Inc. (Tokyo, Japan) using a Procise 492cLC protein sequencer
111 (Applied Biosystems, MA, USA). The N-terminal sequence
112 (NH_2-LPLLTRGRWIVDGATGLRVKLAXVNWVGHL, where X indicates an
113 unidentified amino acid) and the internal amino acid sequence
114 (NH_2-SPLISTNECICITDSHCYP) were obtained (Fig. E2).
115
116 **Determination of human IgE binding by ELISA**
117 Allergen-specific IgE binding was assessed by ELISA. Allergen solution (20 μg/mL)
118 was incubated in a Maxisorp 96-well plate (Thermo Fisher Scientific, MA, USA)
119 overnight at 4°C to coat wells with allergens. After washing 5 times with wash buffer
120 (1% BSA in PBS[−]/0.05% Tween 20), blocking buffer (1% BSA in PBS[−]) was added

121 to each well and incubated for 1 hour at room temperature. After plate washing,
122 5-fold-diluted serum samples or negative control serum (human type AB serum, MP
123 Biomedicals) was added and incubated for 2 hours at room temperature. The plate was
124 then washed 5 times, and 7,000-fold-diluted HRP-labeled goat anti-human IgE antibody
125 (Novus, CO, USA) was added as a secondary antibody and incubated for 40 minutes at
126 room temperature. Substrate solution was prepared with TMB Substrate Reagent Set
127 (BD Biosciences, NJ, USA) and added to each well. After 15 minutes of incubation, an
128 equal volume of 2 M H_2SO_4 solution was added to each well to stop the reaction.
129 Finally, the optical density (OD) at 450 nm was measured using a microplate reader
130 (Tecan, Männedorf, Switzerland). Specific IgE binding was defined as positive when
131 the OD_{450} value was more than 2 times the OD_{450} value of the negative control.
132

133 **Basophil activation test (BAT)**

134 Heparinized whole blood was analyzed using an Allergenicity Kit (Beckman Coulter,
135 CA, USA) according to the manufacturer's instructions. Briefly, indicated
136 concentrations of allergen solutions and 20 μL of an antibody cocktail composed of
137 anti–human CD3-PC7, anti–human CRTH2-FITC, and anti–human CD203c-PE were
138 added to 100 μL of heparinized whole blood. The samples were immediately incubated
139 in a 37°C water bath for 15 minutes under shaded light. Next, 100 μL of stop solution
140 and 2 mL of "Fix-and-Lyse mixture" were added to each sample and incubated at room
141 temperature for 10 minutes. Cells were collected by centrifugation and analyzed using a
142 BD FACSCanto flow cytometry system (BD Biosciences).
143

144 **Cloning of Cha o 3 cDNA**

145 Total RNA was isolated using PureLink® Plant RNA Reagent (Life Technologies) from
146 the female flower of Japanese cypress. Subsequently, cDNA was synthesized using a
147 Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies).
148 According to the results of Cha o 3 protein sequencing, highly conserved regions were
149 observed in an unnamed protein product of *Vitis vinifera* (accession number
150 CAO65892). Hence, we designed forward
151 (5'-AGAGTAAAGCTAGCATGCGTCAATTGG-3') and reverse
152 (5'-CAGCACAGCACCACTTGGGCTTACTCAC-3') primers based on this
153 information. After PCR amplification, the sequence of the PCR product was determined
154 using a DNA sequencer (3130xl; Applied Biosystems). The second forward
155 (5'-CTGGTGTCATGGTCATATTG-3') and reverse
156 (5'-GAGTCAGTGATGCAIATRCAYTC-3') primers were designed based on the
157 identified cDNA sequence and information for the similar protein in *V. vinifera*,
158 respectively. PCR amplification and sequencing of the cDNA were performed again to
159 determine the internal sequence of the Cha o 3 cDNA. Subsequently, the 5' and 3'
160 regions of the Cha o 3 gene were identified by 5'- or 3'-RACE using a GeneRacer kit
161 (Invitrogen, MA, USA) and a 5'/3' RACE kit (Roche, Basel, Switzerland). 5'-RACE
162 was performed using the primers 5'-CTCCGGATTGTTGTGCTCGATTC-3' and
163 5'-CAGCACAGCACCACTTGGGCTTACTCAC-3'. 3'-RACE was performed using
164 the primer 5'-GTTCATCCTGCATTTCCACTCAAGG-3'.
165
166 **In silico analysis of the Cha o 3 protein**
167 Homology was assessed by searching the Protein Sequence Data Banks using
168 NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The signal peptide was

169 predicted using GENETYX software, ver. 9.0.2. A Pfam-A search was conducted to
170 predict the family of protein Cha o 3 (http://pfam.xfam.org/search).
171
172 **Quantification of allergen-specific IL-5 production by PBMCs**
173 PBMCs were separated by Ficoll-Paque density gradient centrifugation from
174 whole-blood samples obtained from 47 patients in the spring, as described previously.[E5]
175 PBMCs (1×10^6) were stimulated with 10 μg/mL of Cry j 1, Cry j 2 (Hayashibara Co.,
176 Ltd., Okayama, Japan), Cha o 1, rCha o 2, or Cha o 3. After 72 hours of culturing, the
177 supernatant was collected and stored at −80°C until cytokine analysis. Finally, the
178 presence of allergen-specific IL-5 in the supernatant was measured using an IL-5 ELISA
179 kit (R&D Systems, MN, USA).
180
181 **Lymphocyte stimulation test**
182 PBMCs (2×10^5) were stimulated with 10 μg/mL of Cha o 1, rCha o 2, Cha o 3, or SBP
183 (Hayashibara) for 72 hours. Incorporation of [^{3}H]-thymidine (PerkinElmer, MA, USA)
184 was assessed using a standard method, as described previously.[E5] The results are shown
185 as the stimulation index (SI), where SI = (mean cpm in triplicate cultures stimulated
186 with antigen)/(mean cpm in unstimulated triplicate cultures). An SI value >2 was set as
187 a positive response. Expected levels of contaminated LPS in the Cha o 1 and rCha o 2 in
188 the assay system were undetectable and that in the Cha o 3 was <0.05 EU/mL. It was
189 confirmed that these levels of LPS did not induce lymphocyte stimulation in this
190 system.
191
192

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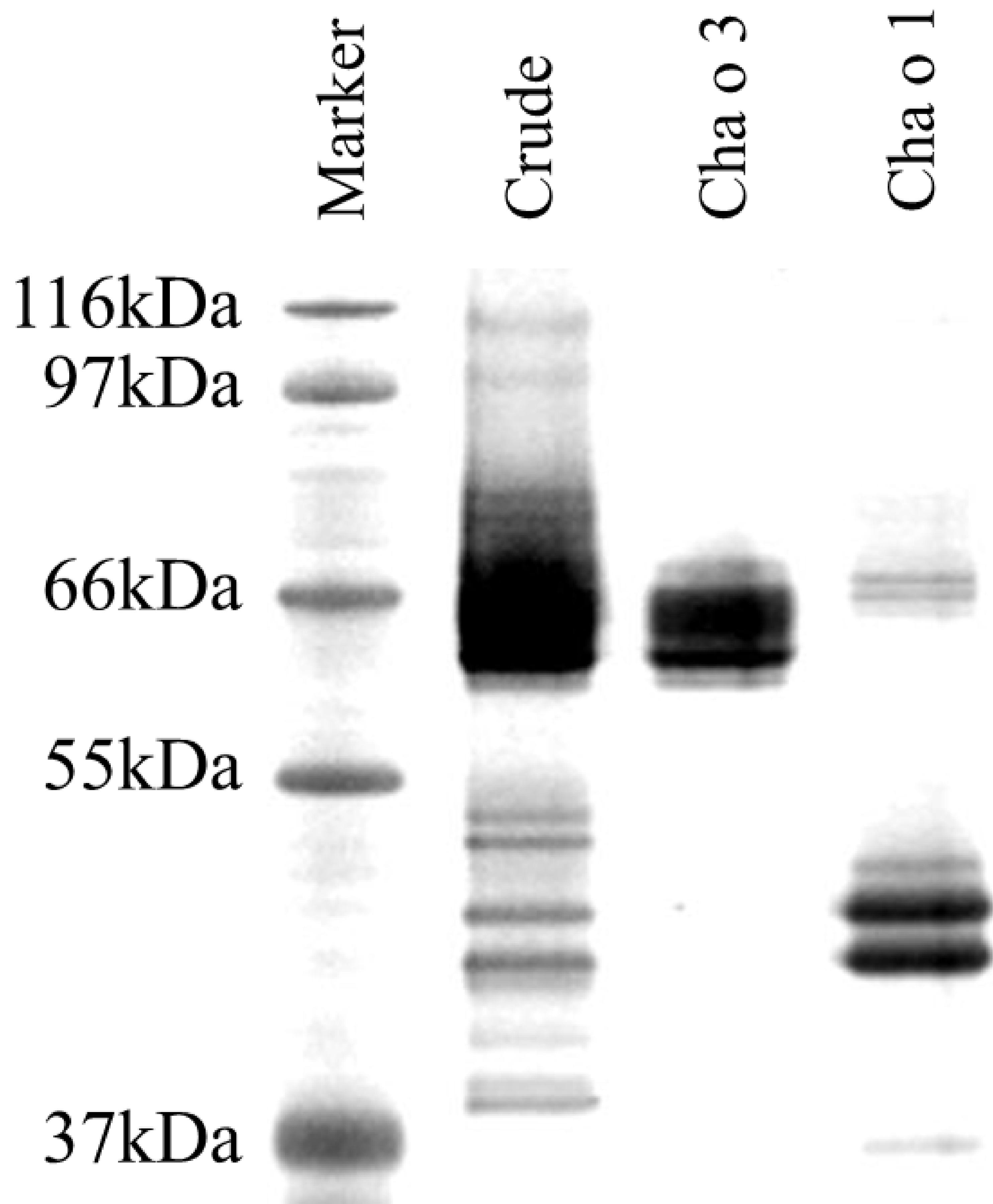
A
Marker
Crude
Cha o 3
Cha o 1
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97kDa
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55kDa
37kDa

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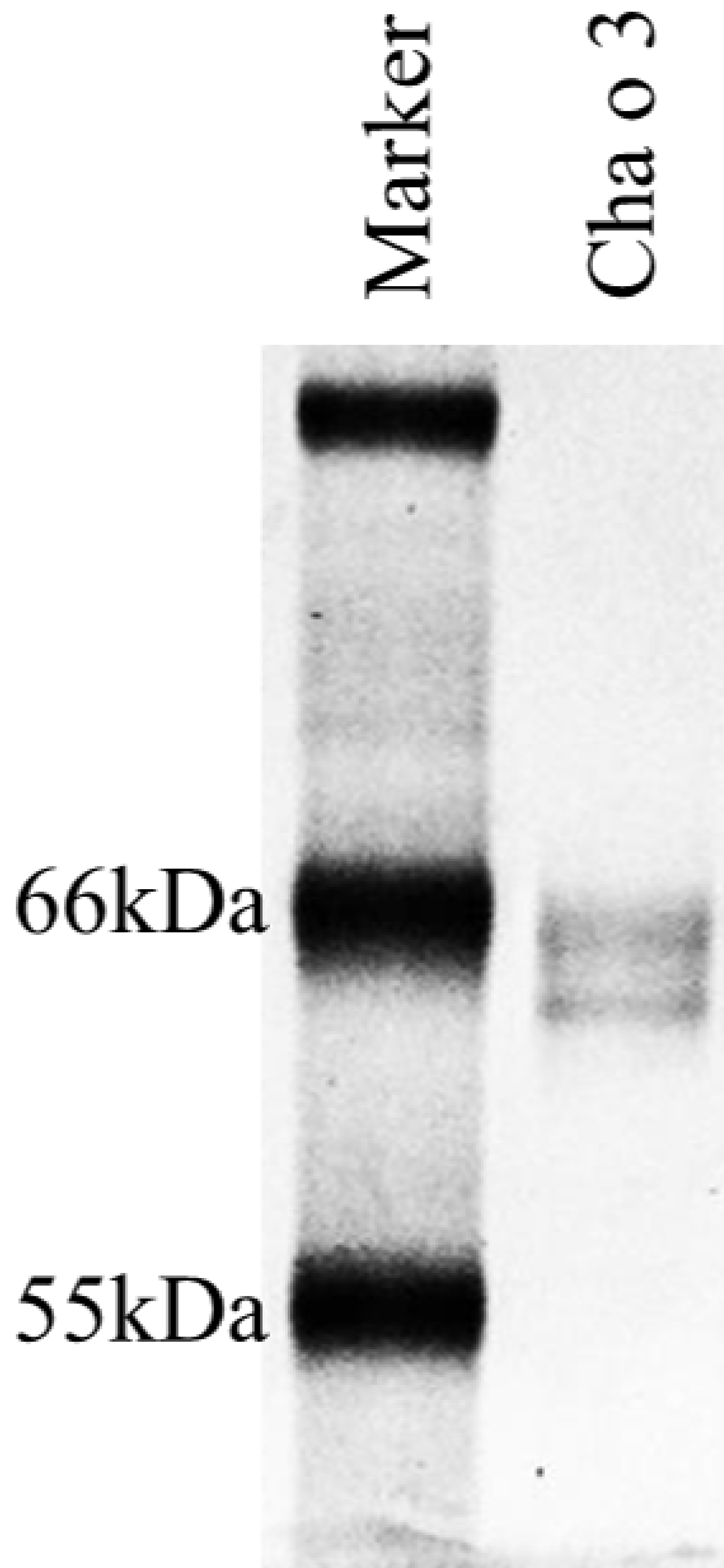
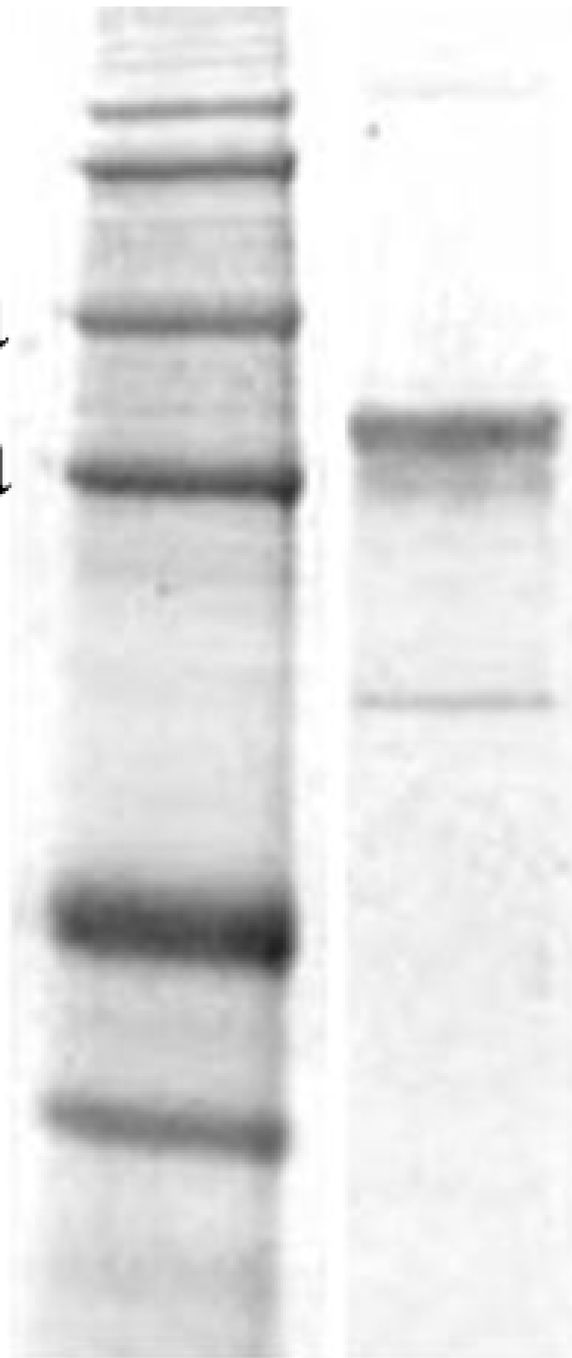
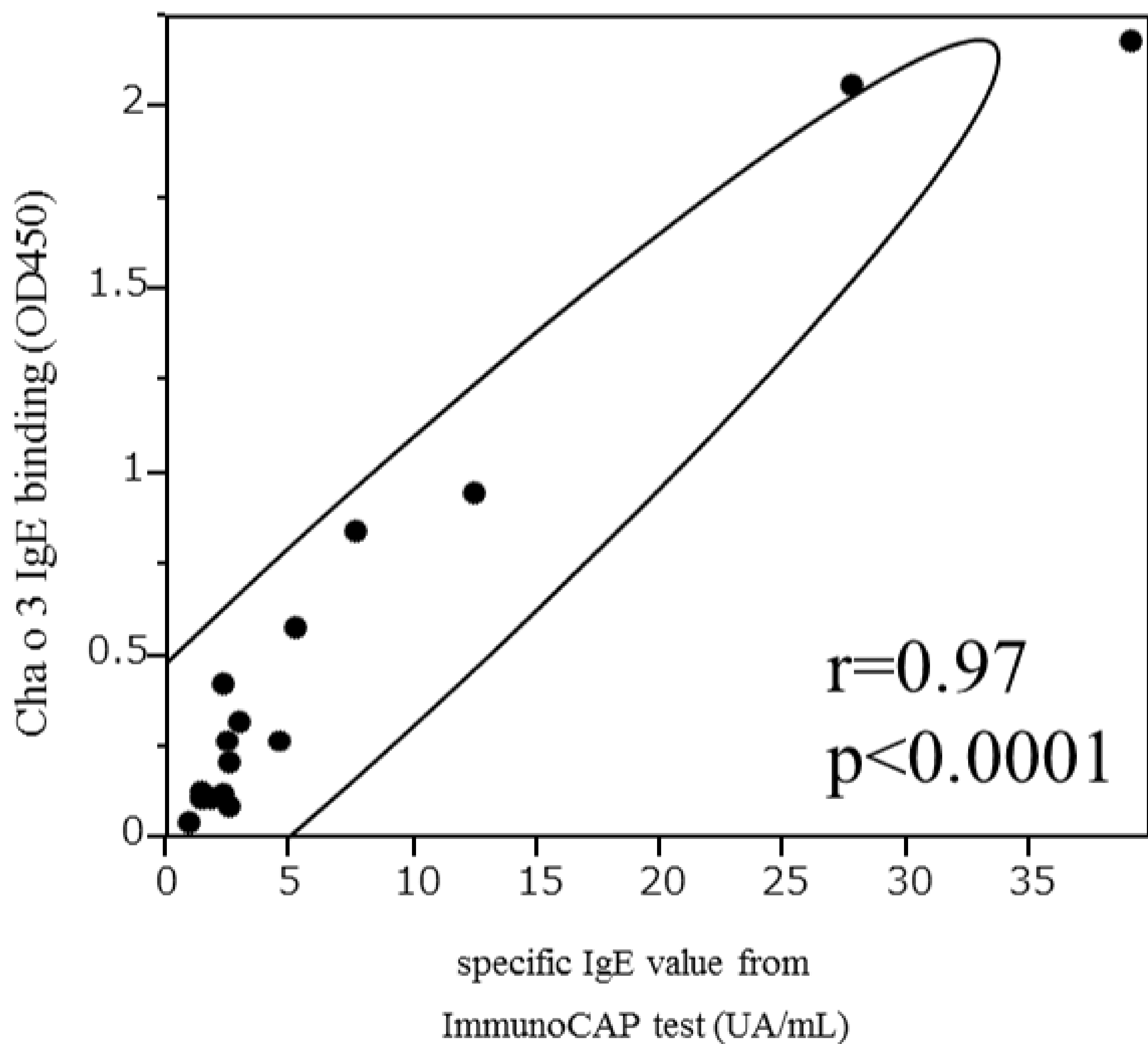


C



D



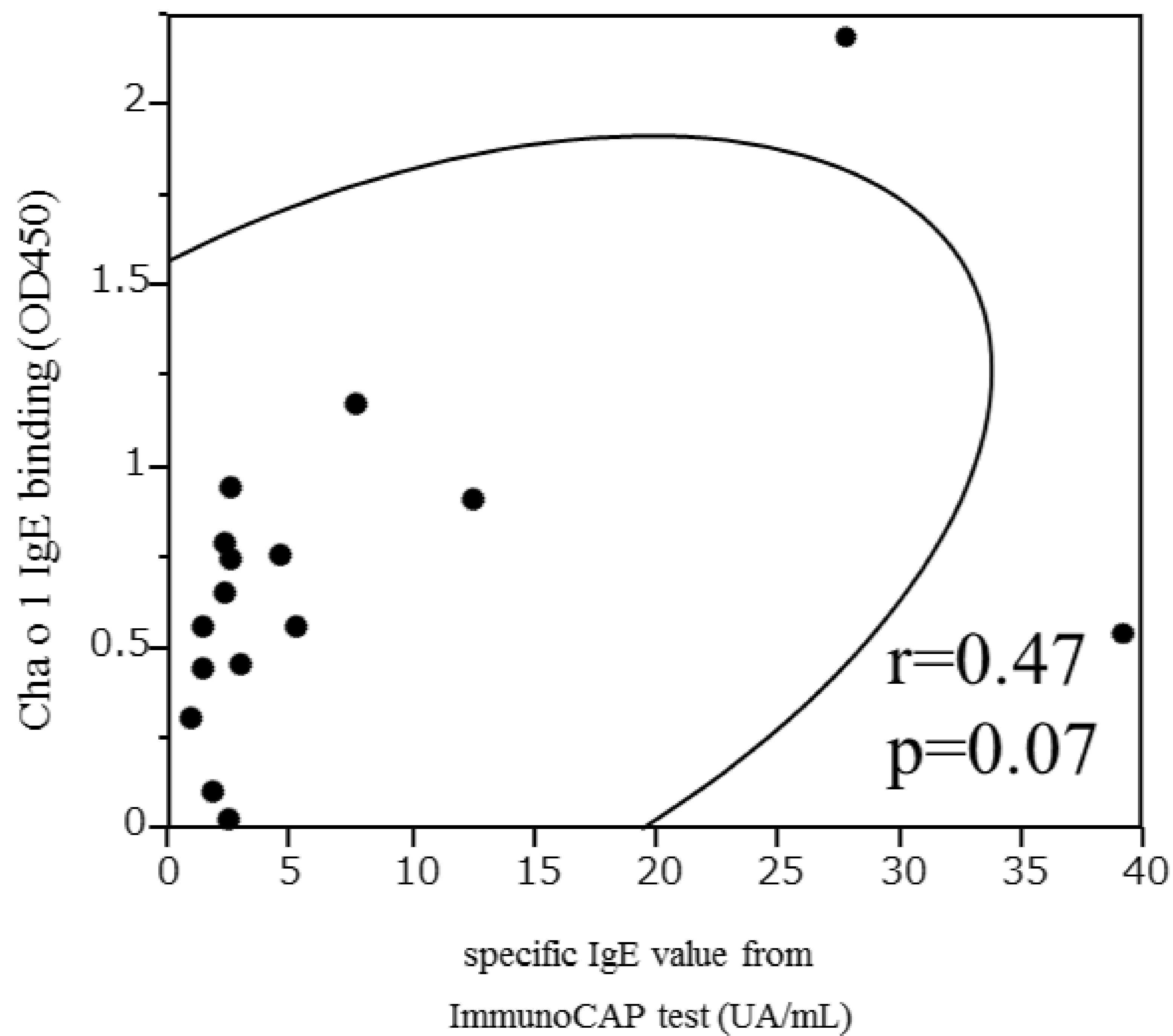
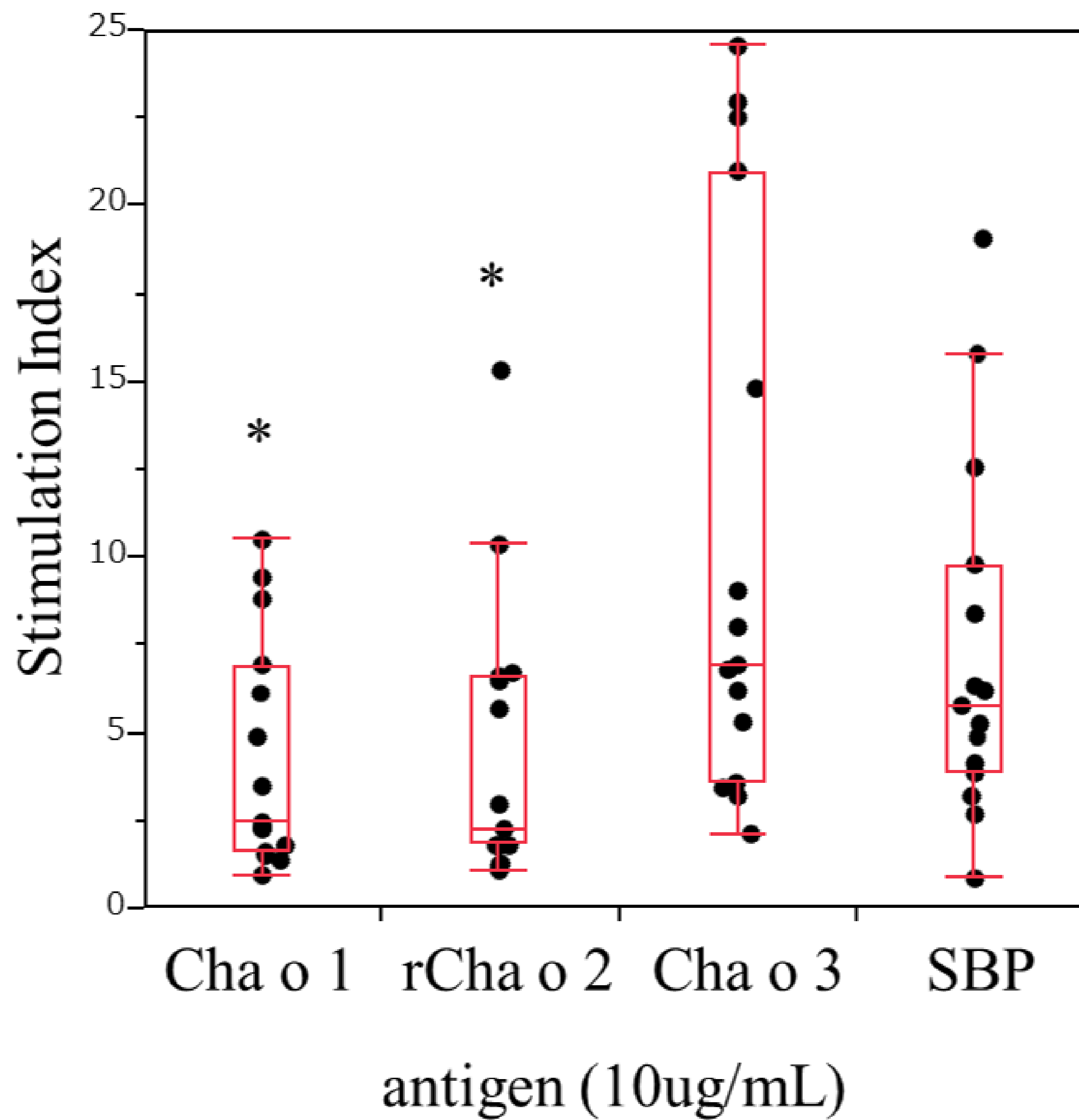
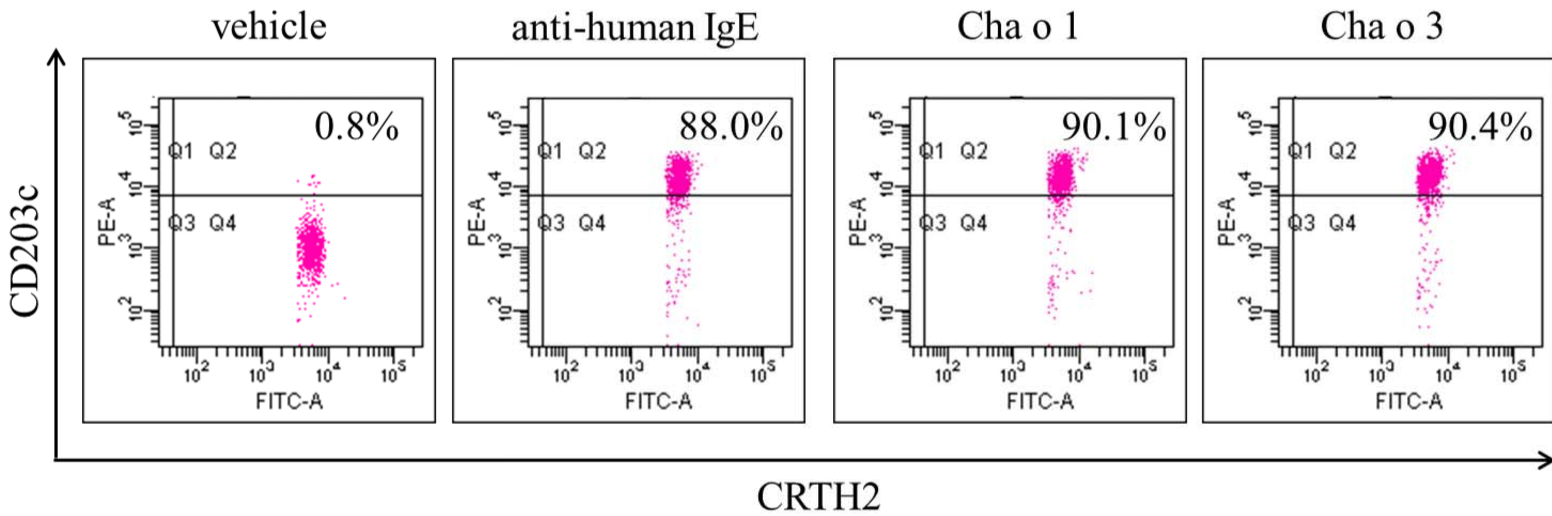
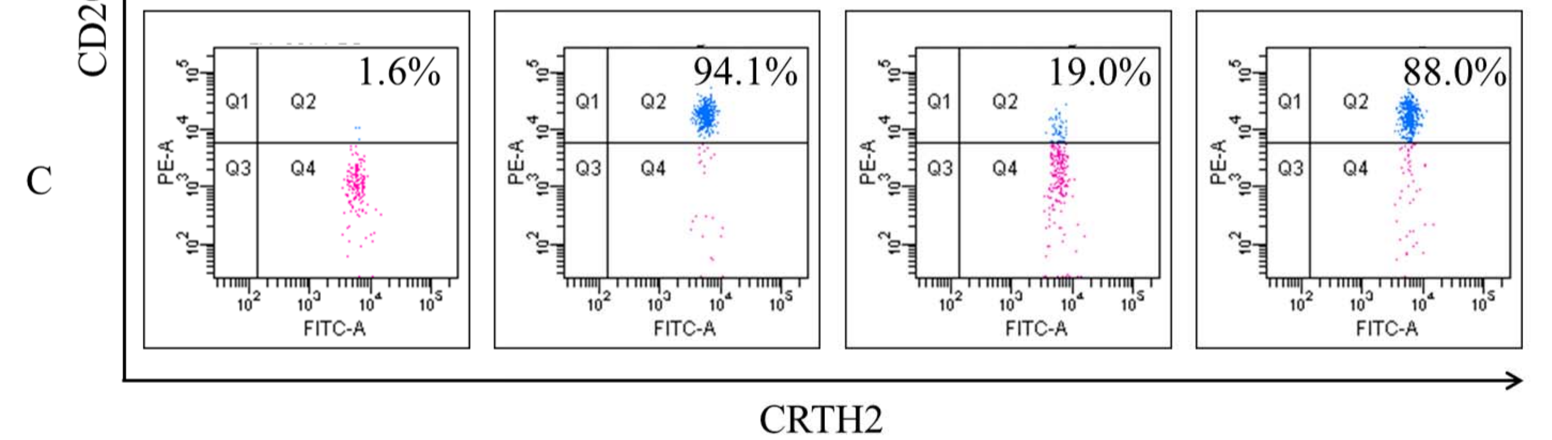

E
Cha o 1 IgE binding (OD450)
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
r=0.47
p=0.07
specific IgE value from
ImmunoCAP test (UA/mL)

Fig. 2



positive ratio	66.7%	53.3%	100%	93.3%


A
vehicle
anti-human IgE
Cha o 1
Cha o 3
0.8%
88.0%
90.1%
90.4%
Q1
Q2
Q3
Q4
PE-A
FITC-A
CD203c
CRTH2


vehicle
SBP
Cha o 1
Cha o 3
B
C
3.1%
87.7%
94.4%
21.0%
1.6%
94.1%
19.0%
88.0%
Q1
Q2
Q3
Q4
PE-A
FITC-A
CD203c
CRTH2

```
1    AAAAAAAGATAAAAATTCGATCTTTAGGGTTTAATGACATCGGCGGGTGTGGCGCCGACG   60
      K  K  R  *  K  F  D  L  *  G  L  M  T  S  A  G  V  A  P  T

61   GCGCTACGATTATTAACAGCGTTGCTATTGCTGCTGGTGGCGGCGCCCTCACATTCGTTG   120
      A  L  R  L  L  T  A  L  L  L  L  L  V  A  A  P  S  H  S  L

121  CCGCTACTGACACGTGGAAGGTGGATCGTGGATGAGGCAACGGGGCTTAGAGTAAAGCTA   180
      P  L  L  T  R  G  R  W  I  V  D  E  A  T  G  L  R  V  K  L

181  GCATGCGTAAACTGGGTGGGCCACCTGGAGCCCGGGCTACCGGAAGGGCTCAACCGGTTG   240
      A  C  V  N  W  V  G  H  L  E  P  G  L  P  E  G  L  N  R  L

241  CCGGTTGCCACAGTAGCGCACACCATTAGCTCCCTGGGCTTCAATTGCGTGCGCCTAACC   300
      P  V  A  T  V  A  H  T  I  S  S  L  G  F  N  C  V  R  L  T

301  TACTCCATCCACATGCTCACCAGGACAAGCTACACCAATGCGACCGTGGCGCAGACGTTT   360
      Y  S  I  H  M  L  T  R  T  S  Y  T  N  A  T  V  A  Q  T  F

361  GCACGGCTAAATCTGACCGAAGCGGCCTCGGGAATCGAGCACAACAATCCGGAGTTGCTT   420
      A  R  L  N  L  T  E  A  A  S  G  I  E  H  N  N  P  E  L  L

421  GACTTGGGCCACGTGGCTGCGTATCATCATGTCGTGGCGGCACTATCGGAGGCTGGTGTC   480
      D  L  G  H  V  A  A  Y  H  H  V  V  A  A  L  S  E  A  G  V

481  ATGGTCATATTGGACAACCATGTGAGTAAGCCCAAGTGGTGTTGTGCTGTGGACGACGGC   540
      M  V  I  L  D  N  H  V  S  K  P  K  W  C  C  A  V  D  D  G

541  AACGGTTTTTTTGGTGACCGTTACTTCAACCCCAACACGTGGGTCGAAGGGCTTGGTCTC   600
      N  G  F  F  G  D  R  Y  F  N  P  N  T  W  V  E  G  L  G  L

601  ATGGCCACGTATTTCAACAACACGCCCAATGTTGTCGCCATGTCGCTTCGGAATGAGCTG   660
      M  A  T  Y  F  N  N  T  P  N  V  V  A  M  S  L  R  N  E  L

661  CGTGGCAACCGATCGACCCCGATATCGTGGTCCAGGCACATGCAGTGGGGTGCCGCTACC   720
      R  G  N  R  S  T  P  I  S  W  S  R  H  M  Q  W  G  A  A  T

721  GTCCACAAGGCCAACCCAAAAGTCCTTGTCATCCTCTCAGGGCTGCAGTTCGACACCGAC   780
      V  H  K  A  N  P  K  V  L  V  I  L  S  G  L  Q  F  D  T  D

781  CTCAGCTTCTTACCGGTCCTGCCGGTCACCCTACCTTTTAAGGAAAAAATTGTTTACGAA   840
      L  S  F  L  P  V  L  P  V  T  L  P  F  K  E  K  I  V  Y  E

841  GGGCACTGGTACTCCTTCGGTGTCCCGTGGCGCACTGGCCTGCCTAACGACGTTTGTAAG   900
      G  H  W  Y  S  F  G  V  P  W  R  T  G  L  P  N  D  V  C  K

901  AACGAGACGGGGCGGTTTAAGAGTAATGTTGGGTTTGTTACATCGTCAGCTAATGCTACT   960
      N  E  T  G  R  F  K  S  N  V  G  F  V  T  S  S  A  N  A  T

961  GCAGCGCCACTTTTTATGAGCGAGTTTGGAATCGATCAGAGATACGTGAATGATAATGAT   1020
      A  A  P  L  F  M  S  E  F  G  I  D  Q  R  Y  V  N  D  N  D

1021 AACAGGTATTTGAACTGTATATTGGCTTATCTGGCTGAGGAGGACTTGGACTGGGCGCTG   1080
      N  R  Y  L  N  C  I  L  A  Y  L  A  E  E  D  L  D  W  A  L

1081 TGGACAATGGGAGGGAGCTATTATTACCGGTCGGACAAGCAGCCCGTTAAAGACTTCGAG   1140
      W  T  M  G  G  S  Y  Y  Y  R  S  D  K  Q  P  V  K  D  F  E

1141 GAGACATATGGATTTTTCAATCATGACTGGTCTCGCATCAGAAACCCTGACTTTATTTCT   1200
      E  T  Y  G  F  F  N  H  D  W  S  R  I  R  N  P  D  F  I  S

1201 AGGCTTAAGGAGATACAACAGCCCATTCAAGACCCTTACTTAGCTCCAGGGCCATATTAC   1260
      R  L  K  E  I  Q  Q  P  I  Q  D  P  Y  L  A  P  G  P  Y  Y

1261 CAGATAATCTACCACCCTGCATCAGGTCTGTGTGTTGAATCCGGCATTGGAAACACAGTT   1320
      Q  I  I  Y  H  P  A  S  G  L  C  V  E  S  G  I  G  N  T  V

1321 CATTTGGGATCATGCCAGAGTGTGAGAAGCAGATGGAACTACGATGCCAGCGTGAAAGGC   1380
      H  L  G  S  C  Q  S  V  R  S  R  W  N  Y  D  A  S  V  K  G

1381 CCAATTGGGCTAATGGGAAGTTCATCCTGCATTTCCACTCAAGGAAATGGGTTGCCTGCA   1440
      P  I  G  L  M  G  S  S  S  C  I  S  T  Q  G  N  G  L  P  A

1441 ATTATGACAGAAAATTGCTCCGCACCCAACAACACTCTGTGGAGTACAGTCTCCTCTGCA   1500
      I  M  T  E  N  C  S  A  P  N  N  T  L  W  S  T  V  S  S  A

1501 CAGCTGCAGCTGGGCACTAGAGTTCTTGGTAAAGATGGGAAAGAGAAGTGGATGTGTTTG   1560
      Q  L  Q  L  G  T  R  V  L  G  K  D  G  K  E  K  W  M  C  L

1561 GATGGGAGTAAAAGTCCTTTGATTTCAACAAATGAATGCATCTGCATTACTGACTCTCAC   1620
      D  G  S  K  S  P  L  I  S  T  N  E  C  I  C  I  T  D  S  H

1621 TGCTACCCAAAATTGAATCCAGAAAAGCAGTGGTTTAAAGTCATAACTACCAACAAGCAA   1680
      C  Y  P  K  L  N  P  E  K  Q  W  F  K  V  I  T  T  N  K  Q

1681 TTGTTGCATCAACTTCAATTGTAA   1704
      L  L  H  Q  L  Q  L  *
```

B

```
[Medicago truncatula] PVDVISNGIKSMGFNCVRLTWPILLLTNDTLSS-LTVRQSFQNLGLLQSVAAFQSNNPSI
    [Theobroma cacao] PMDVIAKRIVSTGFNCVRLTWPLFLVTNDSLAS-LTVRQSFQRLGLLESIAGIQTNNPSI
[Arabidopsis thaliana] PVDAVAKKIVEMGFNCVRLTWPLDLMTNETLANNVTVRQSFQSLGLNDDIVGFQTNNPSI
              Cha o 3 PVATVAHTISSLGFNCVRLTYSIHMLTRTSYTN-ATVAQTFARLNLTEAASGIEHNNPEL

                      IDVSLIQAFQAVVKSLGDNDVMVILDNHITQPGWCCSNSDGNGFFGDQYFDPNLWIQGLT
                      IDVSLLKAYQAVVCSLGENNVMVILDNHISKPGWCCSNFDGNGFFGDQYFNPDIWITGLT
                      IDLPLIEAYKTVVTTLGNNDVMVILDNHLTKPGWCCANDDGNGFFGDQFFDPTVWVAALK
                      LDLGHVAAYHHVVAALSEAGVMVILDNHVSKPKWCCAVDDGNGFFGDRYFNPNTWVEGLG

                      KMATLFNGVSNVVGMSLRNELRGPKQNVNDWYRYMVQGAEAVHAANPDVLVILSGLNFDK
                      RMATLVNAVTNVVGMSLRNELRGPKQTVNDWYRYMQKGAEAVHSANPDVLVILSGLNYDK
                      KMAATFNGVSNVVGMSLRNELRGPKQNVNDWFKYMQQGAEAVHSANNKVLVILSGLSFDA
                      LMATYFNNTPNVVAMSLRNELRGNRSTPISWSRHMQWGAATVHKANPKVLVILSGLQFDT

                      DLSYIAKRPVNLTFKGKLVFEAHWYAFTDGQAWASGNPNQVCGQVAGNMKRMSGYLVDQG
                      DLSFIRNRPANLTFTGKLVFEVHWYGFTDGQTWVTGNPNQVCGRVANDMMRTSGFLVDQG
                      DLSFVRSRPVKLSFTGKLVFELHWYSFSDGNSWAANNPNDICGRVLNRIGNGGGYLLNQG
                      DLSFLPVLPVTLPFKEKIVYEGHWYSF--GVPWRTGLPNDVCKNETGRFKSNVGFVTSSA

                      ----WPLFVSEFGVDLRGTNVNDNRYLNCFIAYAAELDLDWALWTLV
                      ----YPLFVSEFGVDQRGTNVNDNRYLNCFLGVAAELDLDWALWTLV
                      ----FPLFLSEFGIDERGVNTNDNRYFGCLTGWAAENDVDWSLWALT
                      NATAAPLFMSEFGIDQRYVNDNDNRYLNCILAYLAEEDLDWALWTMG
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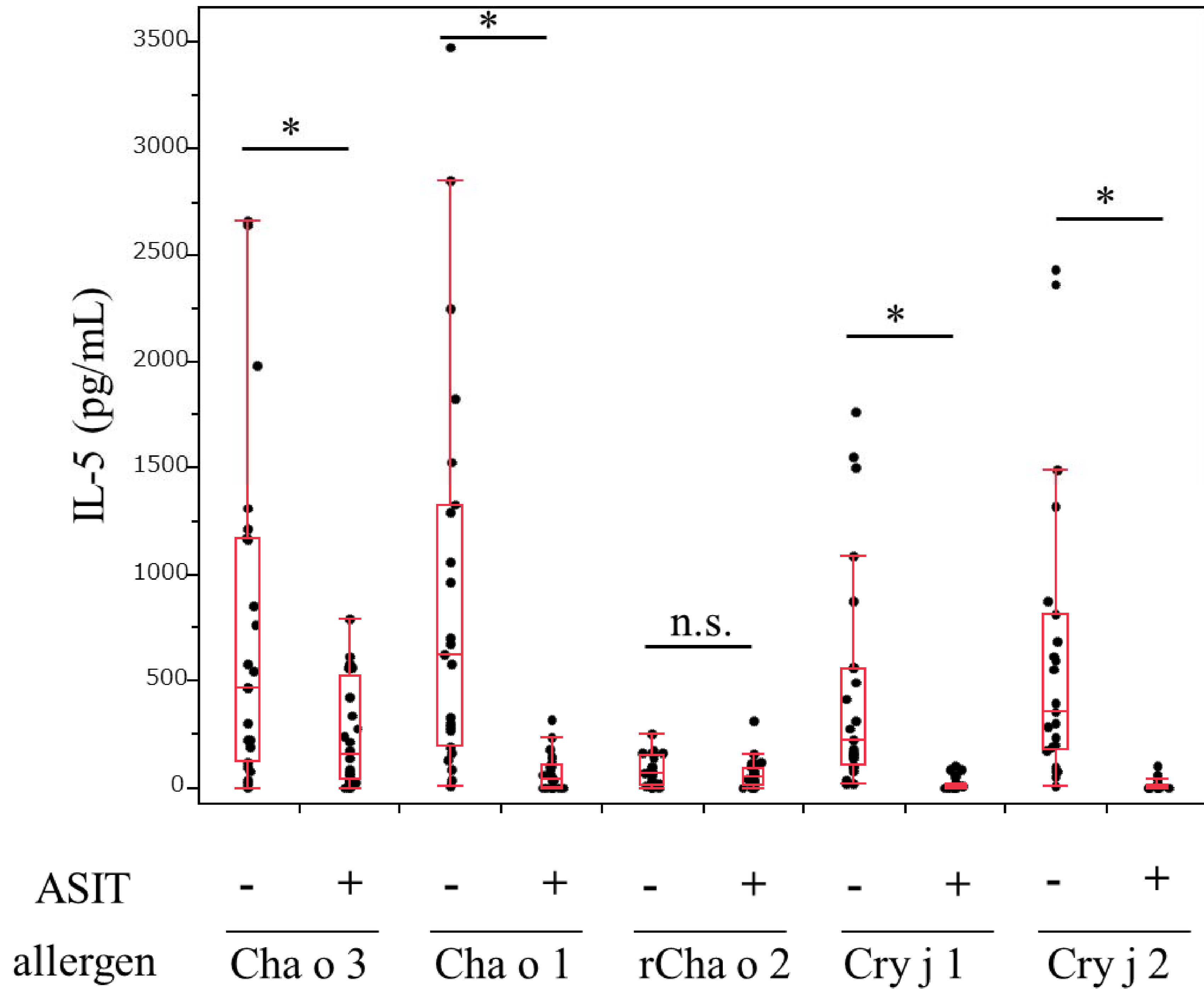
IL-5 (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
*
*
*
*
n.s.
ASIT
-
+
-
+
-
+
-
+
-
+
allergen
Cha o 3
Cha o 1
rCha o 2
Cry j 1
Cry j 2

Table E1

ID	Japanese cedar pollen		Japanese cypress pollen		OD450	
	IgE (U_A/mL)	Class	IgE (U_A/mL)	Class	Cha o 1	Cha o 3
V016	100<	6	39.1	4	+	+
V037	100<	6	27.8	4	+	+
V031	73.6	5	12.5	3	+	+
V043	100<	6	7.63	3	+	+
V045	75.9	5	5.18	3	+	+
V014	36.1	4	4.58	3	+	+
V051	46.7	4	2.92	2	+	+
V026	33	4	2.6	2	+	+
V050	43.7	4	2.6	2	+	-
V047	31	4	2.41	2	-	+
V012	49.2	4	2.35	2	+	+
V042	34.2	4	2.34	2	+	+
V013	8.8	3	1.77	2	+	+
V018	19.5	4	1.43	2	+	+
V040	19.5	4	1.43	2	+	+
V021	21.6	4	0.88	2	+	-
				positive ratio	93.8%	87.5%