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Identification of Novel Allergenic Components from German Cockroach Fecal Extract by a Proteomic Approach

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Key Words
α-Amylase · Allergen · Cockroach

Abstract
Background: Cockroaches produce potent allergens, and cockroach feces are known to be especially rich in allergens. In this study, we analyze the allergenic components from cockroach feces and evaluate allergenicity of recombinant α-amylase identified from fecal extract. ***Methods:*** IgE-reactive proteins from German cockroach fecal extract were analyzed by proteomic analysis and immunoblotting. Recombinant α-amylase was produced and its allergenicity was evaluated by ELISA. ***Results:*** Analysis of German cockroach fecal extracts identified 12 IgE-reactive components. Most of these allergens were found to be digestive enzymes such as α-amylase, trypsin, chymotrypsin, metalloprotease, and midgut carboxypeptidase A, but the identity of 3 IgE-reactive proteins is still unknown. Glycinin-like proteins, which were likely derived from the cockroach diet, were also identified. German cockroach α-amylase shares the highest identity with pig α-amylase (55.8%), followed by mite group 4 allergens (Blo t 4, 50.4%; Der p 4, 49.8%; Eur m 4, 47.4%). In this study, recombinant α-amylase from German cockroach was expressed, and its allergenicity was examined by ELISA. Specific IgE against recombinant amylase was detected in 41.4% (12/29) of serum samples from German cockroach-sensitized subjects. Recombinant α-amylase was able to inhibit 55% of specific IgE to German cockroach whole-body extract. ***Conclusions:*** Amylase was found to be an important novel allergen in cockroach feces. It is hoped that recombinant α-amylase will be useful for further studies and clinical applications.

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Introduction

Cockroach allergy is one of the most common causes of allergic disorders and is known to be strongly associated with the development of asthma. The Allergen Nomenclature Subcommittee (www.allergen.org) has officially listed 10 different groups of proteins as cockroach allergens, and 6 allergens from German cockroaches have been described in the literature to elicit IgE responses in genetically predisposed individuals [1]. All 6 allergens (Bla g 1, 2, 4, 5, 6, and 7) that have been tested to date exhibit a low degree of IgE reactivity and frequency among Korean cockroach-sensitized subjects [2–7]. Studies from the USA also reported that no purified German cockroach allergens identified by the time of publication were immunodominant, and serum samples from approximately 36% of cockroach-allergic patients did not react to the purified allergens (Bla

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g 1, 2, 4, 5, and 7) [8, 9]. In a recent study, Chuang et al. [10] identified 10 new IgE binding proteins by proteomic analysis of German cockroach whole-body extract.

The debris, secretions, and cast-overs of cockroaches are thought to be important sources of cockroach allergens, and it is known that cockroach feces are an important sensitizing agent [11, 12]. In this study, we describe the proteomic analysis of German cockroach fecal extracts. At least 10 IgE binding components were identified in this analysis. A recombinant protein of one of the new allergens, α-amylase, was produced, and its allergenicity was investigated.

Materials and Methods

Serum Samples

Sera were obtained from 29 patients (age range 3–57 years; average 23) who visited the Allergy Clinic at Yonsei University College of Medicine, Seoul, Korea. The diagnosis of allergic disease was based on history and a skin prick test. All sera were tested for the presence of IgE antibodies to *Blattella germanica* using the ImmunoCAP system (Phadia, Uppsala, Sweden) (table 1). Serum samples obtained from 19 subjects with no positive reactions by ImmunoCAP to the tested allergens were used as negative controls. Serum samples were collected after obtaining consent from each patient. Experiments using the collected sera were approved by the Institutional Review Board of our institution (4-2009-0180).

Preparation of German Cockroach Fecal Extract

Our cockroach colony was maintained and the preparation of extracts was done at the Arthropods of Medical Importance Resource Bank, Department of Environmental Medical Biology and the Institute of Tropical Medicine, Yonsei University College of Medicine, Seoul, Korea. Mouse food (Cargill Agri Purina, Sungnam, Korea) was used as a diet.

After euthanasia with carbon dioxide gas, cockroach whole-body extract was prepared by pulverizing frozen cockroaches (30 g) in liquid nitrogen. Allergens were extracted with slow overhead stirring at 4°C in phosphate buffered saline, pH 7.4, containing 6 mM 2-mercaptoethanol, 1/1,000 volume of protease inhibitor set III (Calbiochem, San Diego, Calif., USA), and 1 mg/ml of 1-phenyl-3-(2-thiazolyl)-2-thiourea (Sigma-Aldrich, St. Louis, Mo., USA). Extracts were filtered through a syringe filter (0.22 μm, Millipore, Bedford, Mass., USA) after centrifugation at 10,000 g for 30 min at 4°C.

For the preparation of cockroach feces extract, live cockroaches were transferred to a new basket overlaid with white paper and paper cups, and newly defecated feces were collected with fine brushes every morning and stored at –70°C. Fecal extracts (10 g) were prepared using the same method described above for the preparation of whole-body extracts.

Two-Dimensional Gel Electrophoresis and Western Blot Analysis

Two-dimensional gel electrophoresis was carried out after desalting with trichloroacetic acid. One milligram of protein was separated on a 9–17% gradient sodium dodecyl sulfate polyacrylamide gel after isoelectric focusing (pH 3–10) in an 18-cm tube. The separated proteins were stained with Coomassie Brilliant Blue R250 or electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (0.45 μm, Millipore).

For the identification of allergens, IgE-reactive proteins were probed with mouse monoclonal antibodies (1:1000) (10A6 for Bla g 1 detection, and 7C11 for Bla g 2 detection) (Indoor Biotechnologies Inc., Wiltshire, UK) or pooled serum of 38 subjects (1:4) for 1 h for monoclonal antibodies and overnight for serum samples. Blots were incubated with alkaline phosphatase (1:1000) conjugated goat anti-mouse IgG or with mouse monoclonal anti-human IgE (Sigma-Aldrich) for 1 h. Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega, Madison, Wisc., USA).

N-Terminal Sequencing

Proteins transferred onto PVDF membrane and visualized by Western blotting and Ponceau S staining were excised and subjected to Edman degradation using a Procise 491HT protein sequencer (Applied Biosystems, USA) at the Korea Basic Science Institute.

LC-Coupled ESI-MS/MS Analysis

Some proteins were not able to be identified by N-terminal sequencing, possibly due to N-terminal blocking. These were identified by LC-coupled ESI-MS/MS analysis at ProteomeTech (Seoul, Korea).

Molecular Cloning of α-Amylase

Total RNA was isolated from frozen cockroaches using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, Calif., USA), which was used according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using AMV reverse transcriptase (Promega) and an oligo(dT_{18}) primer. A Champion™ pET Directional TOPO Expression kit (Invitrogen) was utilized for directional cloning into a pET200/D-TOPO vector. Four bases added to the forward primer (CACC) anneal to the overhang in the vector (GTGG) and orient the PCR product. RT-PCR was carried out using forward (5′-CACCCAAAAAGATCCTCACGTTTG-3′) and reverse (5′-CTAtGAACAAGTGCAGTGAGAT-3′) primers, which were designed based on the α-amylase sequence obtained from GenBank (accession No. DQ355516), which was previously deposited by Chuand and Chow from Taiwan.

In the resultant sequence, 18 amino acids were truncated from the original sequence and 37 additional vector-derived amino acids were added. Ultimately, a 534-amino-acid sequence, including a partial pro- and a mature protein, was cloned for expression. Sequencing for cloning confirmation was done by Solgent (Daejeon, Korea).

Expression and Purification of Recombinant α-Amylase

Expression of recombinant α-amylase was induced in *Escherichia coli* BL21 (DE3) cells with 1 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) at 37°C for 4 h. Recombinant protein was purified using affinity column chromatography with Ni-NTA agarose (Qiagen, Valencia, Calif., USA), and protein concentrations were measured by Bradford assay (Biorad, Hercules, Calif., USA) using bovine serum albumin (BSA) as a standard. Purified proteins were separated on a 12% SDS-PAGE gel under reducing conditions and stained with Coomassie Brilliant Blue R250.

Table 1. Clinical features of each enrolled subject

Subject	Gender	Age years	Symptom/diagnosis	Specific IgE to German cockroach (class)	Total IgE kU/l
S01	M	38	allergic rhinitis; asthma	1.95 (2)	ND
S02	M	49	asthma; chronic urticaria	1.22 (2)	ND
S03	M	53	allergic rhinitis; asthma	21.0 (4)	1114
S04	M	8	allergic rhinitis; sinusitis	16.1 (3)	389
S05	M	25	allergic rhinitis; asthma	2.17 (2)	685
S06	M	14	asthma; acute attack	6.02 (3)	474
S07	M	57	allergic rhinitis	13.0 (3)	ND
S08	M	7	allergic rhinitis; asthma	3.11 (2)	415
S09	M	7	allergic rhinitis; asthma	1.58 (2)	234
S10	M	16	allergic rhinitis	2.34 (2)	224
S11	M	13	asthma	1.72 (2)	1,494
S12	M	9	allergic rhinitis	2.09 (2)	3,600
S13	F	10	allergic rhinitis; atopic dermatitis	2.31 (2)	3,266
S14	M	7	allergic rhinitis	2.62 (2)	2,311
S15	M	8	allergic rhinitis; asthma	1.74 (2)	1,994
S16	F	34	anaphylaxis	1.66 (2)	ND
S17	M	6	allergic rhinitis	6.98 (3)	771
S18	M	27	allergic rhinitis; asthma; chronic urticaria	4.71 (3)	305
S19	F	17	allergic rhinitis	2.76 (2)	443
S20	M	14	allergic rhinitis	3.63 (3)	406
S21	M	28	allergic rhinitis	9.45 (3)	926
S22	M	41	allergic rhinitis; atopic dermatitis	6.68 (3)	3,769
S23	F	5	atopic dermatitis	4.18 (3)	ND
S24	M	31	allergic rhinitis; allergic conjunctivitis; asthma	2.48 (2)	845
S25	M	27	allergic rhinitis; asthma	7.79 (3)	403
S26	M	37	chronic urticaria	8.05 (3)	ND
S27	M	45	allergic rhinitis; allergic conjunctivitis	4.19 (3)	503
S28	M	3	asthma	4.98 (3)	81.4
S29	M	28	allergic rhinitis; allergic conjunctivitis; atopic dermatitis	1.45 (2)	2726

* ND = Not determined.

For refolding, purified proteins were dialyzed extensively against refolding buffer (100 mM Tris, pH 8.0, 400 mM L-arginine, 500 mM oxidized glutathione, 5 mM reduced glutathione).

Assessment of IgE Reactivity

Microtiter ELISA plates were coated (100 μl/well) with recombinant protein (2 μg/ml) or cockroach extract (10 μg/ml). A 1:4 diluted serum sample (50 μl) from cockroach sensitized or non-sensitized subjects was added to each well and incubated for 1 h after blocking with 3% skim milk. Diluted biotinylated goat anti-human IgE (1:1,000, 50 μl/well) (Vector, Burlingame, Calif., USA) was added and incubated for 1 h. Streptavidin-peroxidase (diluted 1:1,000, 50 μl/well) was then added and was incubated for 30 min. Rinsing with PBS containing 0.05% Tween 20 was performed at least three times between each incubation step. For color development, 3,3′,5,5′-tetramethylbenzidine substrate solution (100 μl/well) (Kirkegaard Perry Laboratories, Gaithersburg, Md., USA) was added, and the reaction was stopped by the addition of 0.5 M H_2SO_4 (100 μl/well). The absorbance at 450 nm was measured, and the mean plus 2 SD of the sera from non-sensitized subjects was used as the cutoff value.

For inhibition, extract (10 μg/ml) in coating buffer (50 mM carbonate buffer, pH 9.6) was coated onto each well. After blocking, the wells were incubated with selected serum samples (1:4, a pooled sera of 2 subjects) that had been preincubated for 2 h at room temperature and overnight at 4°C with solutions containing various concentrations of recombinant protein or extracts. IgE antibodies were detected as described above. The inhibition assay was conducted in duplicate.

Results

Proteomic Analysis of German Cockroach Fecal Extract

Bla g 1 and Bla g 2 were identified with monoclonal antibodies. Bla g 4, Bla g 5, and Bla g 7 were not probed because they are not excreted proteins. Bla g 2 was identifiable as a 36-kDa protein with a pI of 4.3, but only de-

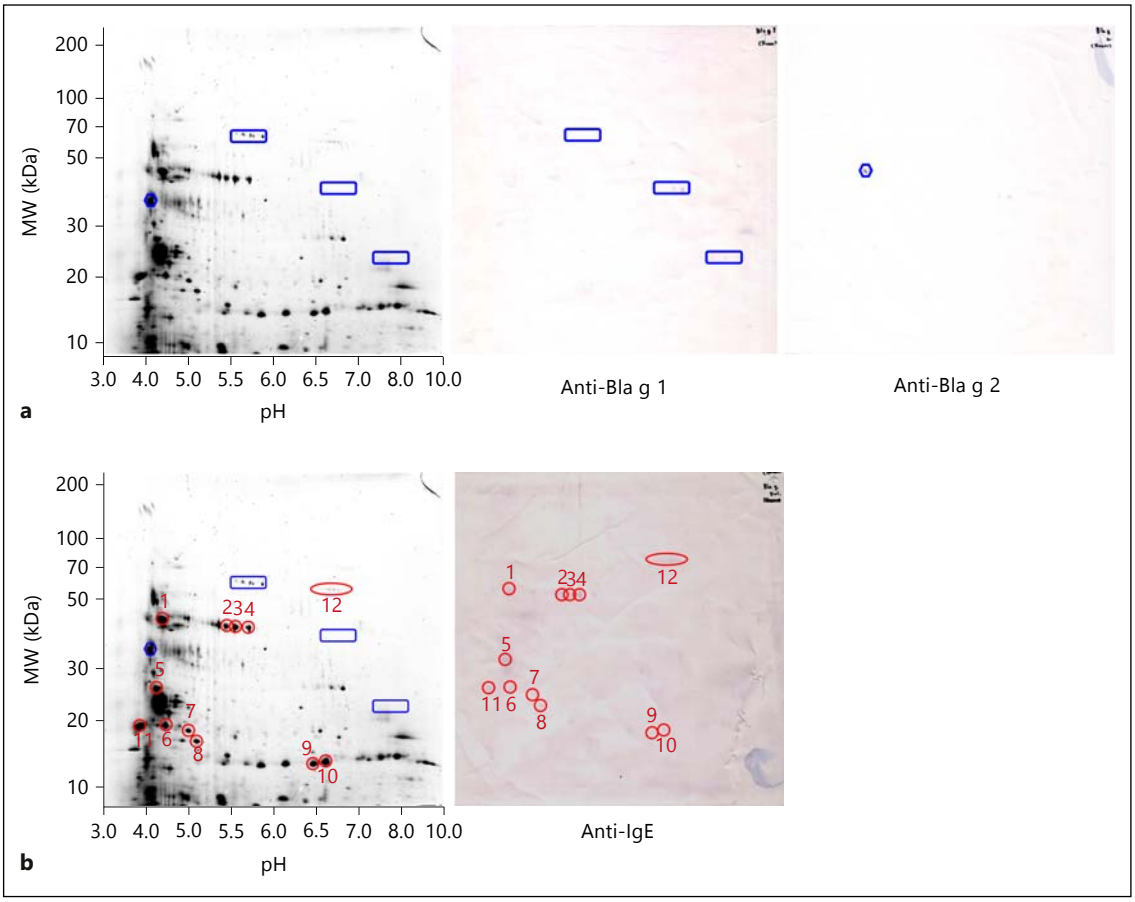

Fig. 1. German cockroach fecal protein (**a**) and IgE-reactive protein (**b**) profiles on two-dimensional gels. Two-dimensionally separated proteins were visualized by Coomassie Blue staining and Bla g 1 (marked with rectangles), and Bla g 2 (marked with a hexagon) were probed with monoclonal antibodies (**a**). IgE-reactive components (marked with circles) were probed with a pooled serum from German cockroach-sensitized subjects (**b**).

graded fragments of about 20, 23, and 40 kDa from Bla g 1 were detected.

Interestingly, no IgE antibodies from German cockroach-sensitized patients recognized either Bla g 1 or Bla g 2, but 12 other proteins were identified by these IgE antibodies (fig. 1). These IgE-reactive spots were subjected to Edman degradation, and the N-terminal sequences of 8 spots were obtained. None of the German cockroach allergen identified to date is known to be a protease activity. However, four of them were found to be digestive enzymes, including midgut carboxypeptidase A (spot 5), chymotrypsin (spot 6), astacin-like metalloprotease (spot 8), and trypsin (spot 11) (table 2). Two spots detectable in the fecal extract were found to be homologous to the soybean allergen Ana o 2, which is thought to be a partially digested fragment of a soy food allergen included in mouse diets designed for fat supplementation. No homologous proteins were identified for 2 spots (spots 7 and 12). LC-coupled ESI MS/MS analysis was performed to identify spots 2, 3, and 4. These spots were determined to be isoforms of α-amylases.

Cloning and Sequence Analysis of α-Amylase from German Cockroach

A single fragment of approximately 1.5 kb was produced by RT-PCR, cloned into pET200D/TOPO vector, and sequenced. The cloned cDNA sequence was 99.9% identical to DQ355516 sequence. Only 1 amino acid (proline to serine at position 52) was shown to be different. A GenBank data search revealed that German cockroach α-amylase shared the highest identity with pig α-amylase (55.8% identity), followed by mite group 4 allergens (Blo t 4, 50.4%; Der p 4, 49.8%; Eur m 4, 47.4%). German cockroach α-amylase was found to have low homology with

Table 2. Identification of IgE-reactive spots from German cockroach fecal extract

Spot	Amino acid sequence	Putative identity	Homology	Accession No.	Organism
1	MS score 182*	α-amylase (LC-ESI MS/MS)	–	DQ355516	*B. germanica*
2	MS score 240	α-amylase (LC-ESI MS/MS)	–	DQ355516	*B. germanica*
3	MS score 213	α-amylase (LC-ESI MS/MS)	–	DQ355516	*B. germanica*
4	MS score 166	α-amylase (LC-ESI MS/MS)	–	DQ355516	*B. germanica*
5	A(Q)VSFDQYYRHTEIND	midgut carboxypeptidase A	69.2% (9/13)	EFA09249	*Tribolium castaneum* (red flour beetle)
6	IT(V)NGQTASRR(G)QTP(PY)	chymotrypsin	76.9% (10/13)	ABV32556	*Teleogryllus emma* (emma field cricket)
7	I(S)V(P)GY(G)EN(VD)AA(Y)IX	unknown	–	–	–
8	NGIISTAYRRPDN(D)T(ND)M	astacin-like metalloprotease	64.3% (9/14)	XP_318543	*Anopheles gambiae*
9	GIDETIXTMRLRQNI	glycinin (Ana o 2 homolog)	93.3% (14/15)	AAA33963	*Glycine max* (soybean)
10	GIDETIXTMRLRHNH	glycinin (Ana o 2 homolog)	80.0% (12/15)	AAA33963	*G. max* (soybean)
11	IVFGGEN(S)ANIEDLPYQ	trypsin	100% (15/15)	AAZ78212	*B. germanica*
12	Q(GDA)TIPAXXVF(SL)S(L)P	unknown	–	–	–

* Mascot scores were given when protein was identified by MS/MS analysis.

allergenic amylases from the other insects (mosquito and biting midge), yeast, fungus, bacteria, and barley (13.3–18.4%) (fig. 2). The cDNA sequence obtained in this study was deposited to the GenBank under accession No. KC207403.

Production of Recombinant α-Amylase

The recombinant α-amylase containing an additional 36 amino acids at the N-terminus gave a band with an apparent molecular mass of about 60 kDa on a Coomassie Blue-stained SDS-PAGE gel. This size is similar to that calculated from the deduced amino acid sequence of the recombinant protein (59 kDa, pI 5.756) and larger than the one calculated for native cockroach α-amylase (56.8 kDa, pI 5.704). The yield of the purified recombinant protein was approximately 9 mg/l from *E. coli* culture.

IgE Reactivity and Inhibition Analysis

The unfolded recombinant allergen was detected by IgE antibodies from 12 of 29 German cockroach-sensitized sera tested (41.4%). However, 9 of 29 sera (31.0%) were determined to be positive after refolding (fig. 3). IgE reactivity of 9 double positives to unfolded α-amylase correlated well with IgE reactivity to refolded protein (correlation coefficient = 0.94, by Pearson's correlation analysis).

Recombinant α-amylase was able to inhibit the 55.0% of IgE reactivity at a concentration of 10 μg/ml (when tested with a pooled sera of 2 subjects who displayed double-positive reactions to unfolded and refolded recombinant proteins), whereas crude whole-body extract showed 94.1% inhibition at the same concentration of inhibitor (fig. 4).

Discussion

Proteomics is a powerful tool for the characterization of allergens. Using this approach, we were able to identify at least 9 different IgE-reactive components from German cockroach fecal extracts (table 2). Furthermore, the allergenicity of recombinant α-amylase was examined. German cockroach α-amylase shares 13.3–55.8% amino acid sequence identity with allergenic α-amylases (fig. 2). About 41.4% of cockroach-sensitized patients showed positive IgE reactivity to recombinant α-amylase and a maximum inhibition rate of 55% to whole-body extract (fig. 4). Low inhibition rate may imply the concentration of α-amylase in whole-body extract was not high. Several isoforms of α-amylases were detectable by two-dimensional electrophoresis and IgE immunoblotting, and at least 3 of these isoforms were shown to be IgE reactive. Therefore, we sought to investigate the allergenicity of these different isoforms. IgE reactivity to α-amylase may be a reflection of low IgE titer to the cockroach allergens (table 1). A 64-kDa allergen previously described by Lee et al. [13], which was shown to have 66.7% IgE reactivity among atopic children, was believed to be α-amylase. In a recent study, proteins of about 60 and 70 kDa were strongly recognized by Korean German cockroach-sensitized subjects, and these allergens are now being investigated for identification and characterization [14].

Amylase activity was previously reported from various gut compartments and salivary glands [15]. Moreover, α-amylase is found to be contained in tergal gland secretion, along with phagostimulatory sugars and

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Bla g amylase      ---------MKLFPLVALLVLVVGVLSQKDPHVWDGRSAIVHLFEWKFADIA---------- 43
Aed a 4            --------MKIFVPLLSFLLAGLTT------GLDWWEHGNFYQVYPRSFKDSDGDGIGDL-- 46
Cul n 8            ----MITFKKLTLLLSISCTVLAAPEGVREKDWWETGNFYQVYPRSFMGSDGDGVGDL-- 54
Der p 4            --------------------------KYHNPHFIGNRSVITHLMEWKYDDIG---------- 26
Eur m 4            MLPKIYLKLITVLFISLFVQNNVHCKYHNPHFIGNRCVITHLMEWKYDDIG---------- 51
Blo t 4            ---MIVELIVFSLALTIPYQTLASSPYSDPHFQHNRKVITHLMQWKFVDIA---------- 48
Sus d amylase      ---------MKLFLLLSAFGFCW---AQYAPQTQSGRTSIVHLFEWRWVDIA---------- 40
Sac c glucosidase  --------------------MTISDHPETEPKWWKEATIYQIYPASFKDSNNDGWGDL-- 38
Asp o 2            --------MMVAWWSLFLYGLQVAAPALAATPADWRSQSIYFLLTDRFARTDGSTTATCNT 53
Bac li aA          ---MKQQKRLYARLLTLLFALIFLLPHSAAAAANLNGTLMQYFEWYMPNDGQ--------- 49
Hor v 16           --------MGKKSHICCFSLLLLLFAGLASGHQVLFQGFNWESWKQSGGWYN---------- 44


Bla g amylase      ---------------DECERFLGSKGFAGVQVSPVHENVIISSPF---RPWWERYQLVSY-K 86
Aed a 4            -----------DGVTEKLKYLKDIGMDGVWLSPIFSSPMADFG--------YDISNFR---E 86
Cul n 8            -----------KGITAKVGYLKEIGMDGVWLSPIFDSPMADFG--------YDISNLT---K 94
Der p 4            ---------------DECERFLGPYGYGGVQVSPVNEHAILDR------RPWYERYQPVSY-D 67
Eur m 4            ---------------DECERFLGPYGYGGVQVSPVNEHAILDR------RPWYERYQPVSY-D 92
Blo t 4            ---------------DECERFLGPYGYGGVQVSPVNEHASLDR------HPWYELYQPVSY-R 89
Sus d amylase      ---------------LECERYLGPKGFGGVQVSPPNENIVVTNPS---RPWWERYQPVSY-K 83
Sac c glucosidase  -----------KGITSKLQYIKDLGVDAIWVCPFYDSPQQDMG--------YDISNYE---K 78
Asp o 2            ADRKYCGGTWQGIIDKLDYIQGMGFTAIWITPVTAQLPQTTAYG--DAYHGYWQQDIY-S 110
Bac li aA          --------HWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYDLYDLGEFHQKGT 101
Hor v 16           -----------MMLGKVDDIAAAGVTHVWLPPPSHSVAAEG----------YMPGRLYD-I 83
                                        :   *   : : *

Bla g amylase      LVSRSGDENAFRDMVRRCNNVGIRIYVDVVLNQMSGSWPDAHG--QGGSTAD-TYNLQYP 143
Aed a 4            IQTEYGDLDAFQRLSDKCKQLGLHLILDFVPNHTSDQHEYFKKSVQKDETYK-DFYVWHP 145
Cul n 8            VFPQFGDLSSIDELVAECNKKDIKLILDFVPNHTSDQCEWFKKSIKRDPEYD-NYYIWHP 153
Der p 4            IRTRSGDEQQFRRMVKRCNKAGVRIYVDIVLNHMTGAQSGKGTN---GHHYD-GNTLQYP 123
Eur m 4            LRTRSGDEQQFRRMVKRCNKAGVRIYVDIVLNHMTGGQSGQGTN---GNHYN-GITMQYP 148
Blo t 4            IVSRSGTESQFRDMVHRCNKAGVRIYVDVVLNHMTGPQSGVGID---GTHYD-GNSMQYP 145
Sus d amylase      LCTRSGNENEFRDMVTRCNNVGVRIYVDAVINHMCGSGAAAGTGTTCGSYCN-PGNREFP 142
Sac c glucosidase  VWPTYGTNEDCFELIDKTHKLGMKFITDLVINHCSTEHEWFKESRSSKTNPKRDWFFWRP 138
Asp o 2            LNENYGTADDLKALSSALHERGMYLMVDVVANHMGYDG----------AGSSVDYSVFKP 160
Bac li aA          VRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGAD----------ATEDVTAVEVDP 151
Hor v 16           DASKYGNAAELKSLIGALHGNGVQAIADIVINHRCADYK----------DSRGIYCIYEG 133
                        *           :  .:    * * *:

Bla g amylase      AVPYGPGDFHS--TCTVSN-----YQDPSNVRNCELVGLH----DLNQGSDYVR-GKMIE 191
Aed a 4            GVHGPNN-TKVPPSNWISVFRGSSWEWNEERQEFYLHQFLKEQPDLNYRNPAVV-EEMKN 203
Cul n 8            GKPNPDGGRNLPPTNWVSAFRSSAWEWNEERGEYYLHQFLAEQPDLNYRNPKVV-ETMKS 212
Der p 4            GVPFGPNDFHGHESCPTQDLEIHDYTNPKEARNCRLSGLR----DLKQQSEYVR-QKQVD 178
Eur m 4            GVPFGPNDFHGHESCQTKDLEIHNYSDRNEARNCRLSGLR----DLKQQSEYVR-KKQVD 203
Blo t 4            GIPFGPNDFHGHESCPTSNLDIQNYDDPTQARNCRLSGLR----DLKQSADYVR-TKQAD 200
Sus d amylase      AVPYSAWDFNDG-KCKTASGGIESYNDPYQVRDCQLVGLL----DLALEKDYVR-SMIAD 196
Sac c glucosidase  PKGYDAEGKPIPPNNWKSFFGGSAWTFDETTNEFYLRLFASRQVDLNWENEDCRRAIFES 198
Asp o 2            ----------FSSQDYFHPFCLIQNYEDQTQVEDCWLGDNTVSLPDLDTTKDVVKNEWYDW 211
Bac li aA          ADRNRVISGEHRIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGKAW 211
Hor v 16           GTS------DSRLDWGPHMICRDDTKYSDGTANLDTGADYADAPDIDHLNHRVQSELKEW 187
                                                          :

Bla g amylase      YLNHLVDCGVAGFRVDAA------KHMWPADLQYIYSKVNNLNTDHGFPSGARPFFYQEV 245
Aed a 4            VLRYWLDRGVSGFRIDAV------PYLFESD-IIDGRYRNEPESRTTDDPENPAYLVHTQ 256
Cul n 8            VLRFWLSKGINGFRIDAV------PYLFEVAPDANGNYPDEIETNACDDPLSQCYLYHDY 266
Der p 4            FLNHLIDIGVAGFRSDAS------THQWPDDLRSIYSRLHNLNKEF-FPENSQPFIYHET 231
Eur m 4            FLNHLIDIGVAGFRSDAS------THQWPDDLRSIYSQLHTLNKEF-FPENSHPFIYHET 256
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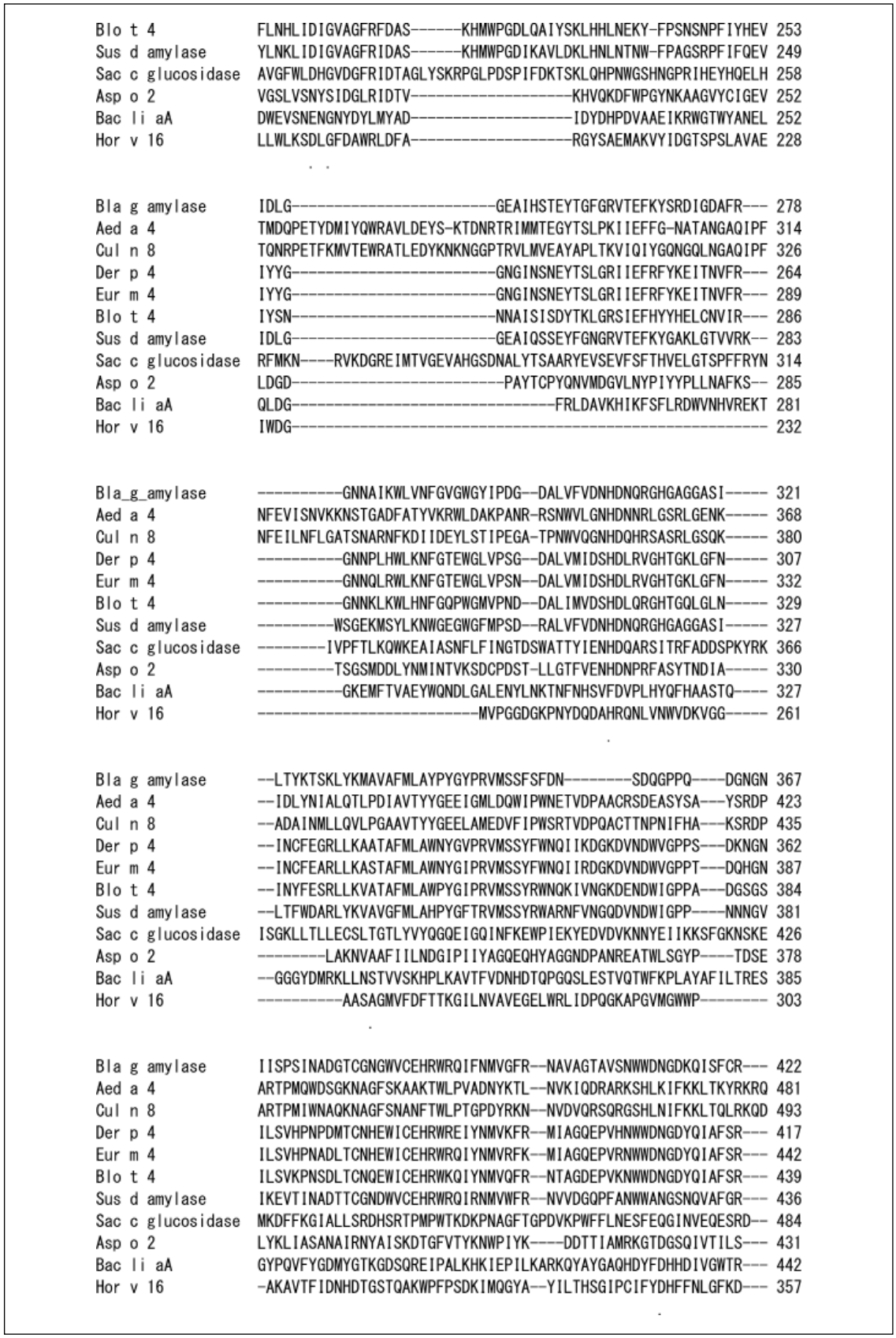
(For legend see page 322.)



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(For legend see page 322.)

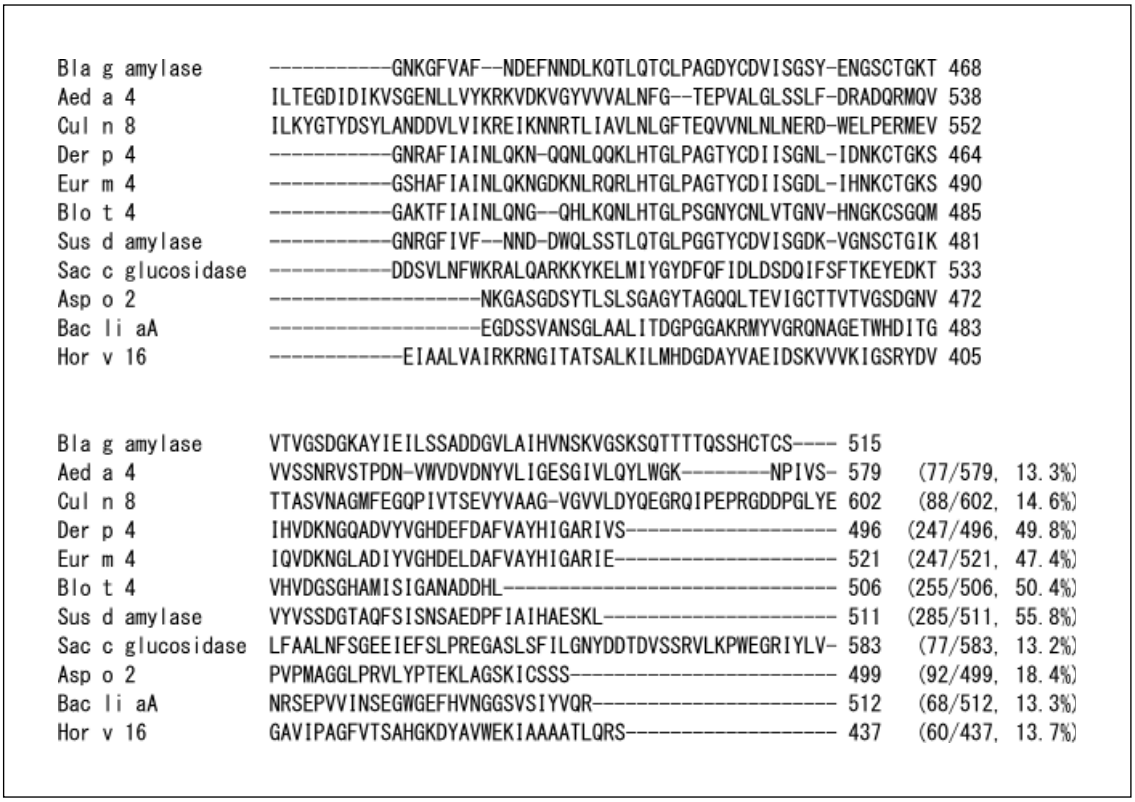
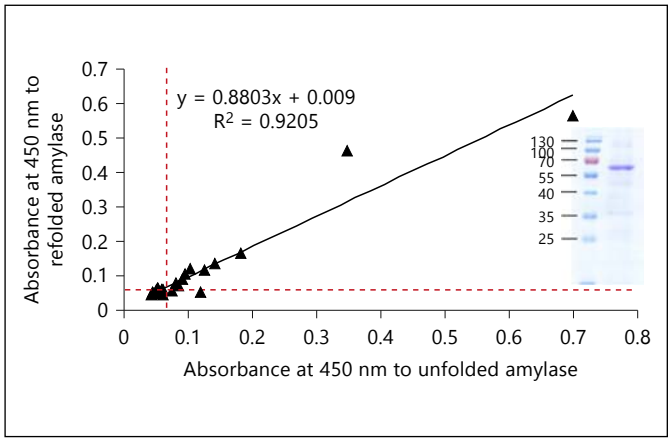


Fig. 2. Amino acid sequence alignment of allergenic α-amylases. Bla g amylase (*B. germanica,* ABC68516); Aed a 4 (*Aedes aegypti,* AAA29352); Cul n 8 (*Culicoides nubeculosus,* ACM40914); Der p 4 (*Dermatophagoides pteronyssinus,* AAD38942); Eur m 4 (*Euroglyphus maynei*, AAD38943); Blo t 4 (*Blomia tropicalis*, AAQ24543); Sus d amylase (*Sus scrofa domestica*, domestic pig, AAF02828); Sac c glucosidase (*Saccharomyces cerevisiae*, baker's yeast, EDV11816); Asp o 2 (*Aspergillus oryzae*, ABY71841); Bac li aA (*Bacillus licheniformis*, CAA26981); Hor v 16 (*Hordeum sativum*, barley, CAA72143).



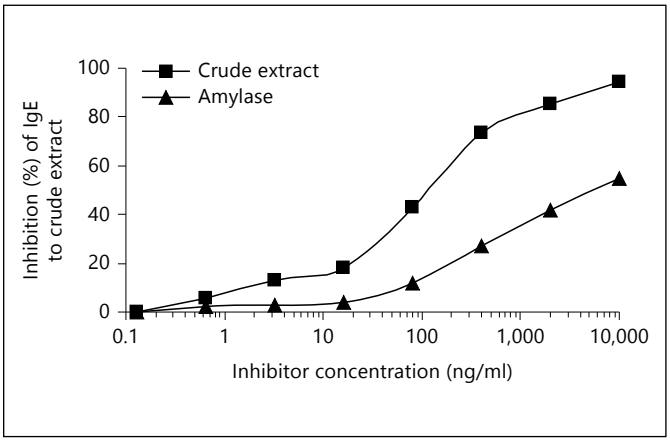


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Fig. 3. IgE reactivity of sera from cockroach-sensitized patients against recombinant cockroach α-amylase and SDS-PAGE analysis of purified protein (**inset**). Dotted lines indicate cutoff values.

Fig. 4. ELISA inhibition of German cockroach-specific IgE with recombinant α-amylase and whole-body extract. Inhibition was performed with a pool of sera from 2 subjects.

phospholipids, which encourage female feeding and arrest the female cockroach in a position favorable for mating [16].

In proteomic analysis, IgE reactive proteins were detected under denaturing conditions. We also compared the reactivity of IgE to α-amylase before and after refolding. However, strong correlation (correlation coefficient = 0.94) was observed, suggesting that conformational epitope does not play an important role (fig. 3). While our results are suggestive of a role for α-amylase in the allergenicity of German cockroach, it will be necessary to conduct a large-scale investigation to better understand α-amylase's role in cockroach allergy.

Differential IgE antibody responses have been reported in cases of mite amylases. Australian Aboriginals were shown to direct major IgE antibodies predominantly to the Der p 4 (α-amylase from *Dermatophagoides pteronyssinus*), with an average 55% of the IgE antibody in each serum, whereas it is a minor allergen in the Perth population, accounting for less than 10% of IgE titer [17]. An unusually higher IgE binding frequency to Blo t 4 (α-amylase from *Blomia tropicalis*) (28%) was reported in allergic subjects from Chengdu, PR China, whereas a low sensitization rate (4%) was shown in the subjects from Singapore [18]. A potential epitope for cross-reactivity of mite group 4 allergens was described from a tertiary structure revealed by homology modeling of Aca s 4, α-amylase from *Acarus siro* for Aca s 4 was recognized by IgE antibodies in house dust mite-sensitive patients [19]. Therefore, it is necessary to investigate the cross-reactivity of mite and cockroach amylases.

Interestingly, the soybean allergen homolog glycinin was identified in fecal extract (table 2). Soybean is a common ingredient in mouse food and was present in the mouse chow fed to our cockroaches. Partially degraded peptide fragments of soybean allergens are thought to be excreted into cockroach feces. It may be possible that some partially degraded food allergens could be excreted and become airborne in indoor environments. Importantly, it is also possible that food allergens, such as soybean allergen, could be contained in the cockroach extracts if cockroaches fed on soybeans were used for the preparation of allergy diagnostics, leading to false-positive diagnoses of cockroach allergies.

As expected, most of the IgE reactive components in the fecal extract were found to be digestive enzymes (table 2). These enzymes could modulate allergic airway inflammation in various ways [20]. Further studies to characterize these proteases and biochemical identification of unknown proteins shown to be IgE reactive are needed for a better understanding and standardization of cockroach extracts.

Bla g 1 and Bla g 2, which are known to be major German cockroach allergens, have not been shown to be IgE reactive in fecal extracts (fig. 1). It is believed that the IgE reactivity of Bla g 1 is very low among Korean subjects [2]. Bla g 2 was thought not to be detected in this study because it is denatured. The allergenicity of denatured Bla g 2 is not high in the Korean population, but the conformational epitope of Bla g 2 is important [3].

In summary, we identified 12 IgE reactive components from German cockroach fecal extracts. Most of the IgE reactive proteins were found to be proteases. Given the nature of the cockroach extract preparation, partially degraded food allergens such as glycinin could be included in the cockroach extract. Recombinant α-amylase, an IgE-reactive protein, was identified and its allergenicity was investigated. Recombinant α-amylase was recognized by 41.1% of the sera from cockroach-sensitized subjects (fig. 3) and showed a maximum inhibition of 55% of IgE reactivity to cockroach whole-body extract (fig. 4). This study will facilitate further characterization of cockroach allergens, which is important for the development of better diagnostic and immunotherapeutic reagents [21].

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