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Characterization of the 41 kDa allergen Asp v 13, a subtilisin-like serine protease from *Aspergillus versicolor*[☆]

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ABSTRACT

Aspergillus versicolor is common on moldy building materials. Asp v 13, the principal allergen is produced by strains collected from across Canada. In this paper, we report a 1833 bp Asp v 13 open reading frame predicted to encode a protein of 403 amino acids in length with three introns. A BLAST search of Asp v 13, a phylogenetic tree calculation and alignment with its homologous proteins from other species indicated that Asp v 13 is a secretory, subtilisin-like serine protease widely distributed in *Aspergillus* species. His-tagged Asp v 13 was over-expressed in *Escherichia coli* and purified using Ni-NTA columns with a yield of 1 mg/L. Based on immuno binding assay of recombinant protein both antibodies developed against the natural protein, and human sera IgE, the recombinant protein was similar to the natural form. Six IgE- and seven IgG-binding epitopes were also identified with selected human sera along the entire amino acid sequence of Asp v 13. Most residues binding these epitopes are exposed on the surface and correspond to charged regions of the molecule.

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1. Introduction

Aspergillus versicolor is common on manufactured wood, paper-faced gypsum wallboard and various cellulose-based materials found indoors in the US, Canada and in various countries in Western Europe and Scandinavia (Miller et al., 2008; Liang et al., 2011). The principle toxin produced by this fungus, sterigmatocystin, has been detected on water-damaged building materials and in dusts from floors (Bloom et al., 2009; Engelhart et al., 2002; Horner et al., 2004; Flannigan and Miller, 2001).

IgG response to *A. versicolor* extracts from sera was fairly common ca. 200 children in Finland as was the case in a larger study of 1000 blood samples in Norway (Korppi et al., 2003; Rydjord et al., 2008). In a German study of 1800 sera collected from children, IgE response to extracts of *A. versicolor* was found in ~2% of the children (Kolossa-Gehring et al., 2007).

Using pre-screened human sera with antibodies to various fungi, two related proteins were discovered with molecular weights 43 and 41 kDa based on SDS electrophoresis, both present in spores and immunogenic in humans, mice and rabbits. The 41 kDa

protein has a pI of 4.5. Based on the partial sequence obtained it was a serine protease with an acid pI (4.5). There are a number of *Aspergillus* and *Penicillium* proteases with overlapping sequences but with different molecular weights and basic pI values. Polyclonal and monoclonal antibodies were developed that were specific compared to a diverse taxonomic array of related and unrelated fungi that commonly occur in the built environment (Liang et al., 2011).

In this paper, we report the use of inverse PCR and RT-PCR to obtain the cDNA sequences encoding Asp v 13. The protein name was approved by the I.U.I.S. allergen nomenclature sub-committee. Recombinant form of Asp v 13 was expressed in *E. coli* that was indistinguishable from Asp v 13 isolated from *A. versicolor* in western blot analyses. Additionally, the three-dimension structure was modeled and its IgE/IgG binding epitopes were screened by epitope mapping with synthesized peptides using the spot peptide technique.

2. Materials and methods

2.1. Sequence determination

The design for the Asp v 13 (<http://www.allergen.org/>) sequence probe followed the three steps PCR protocol described in Shi et al. (2011). Briefly this involved the use of PCR, degenerate primers and genomic DNA template to reveal the partial genomic sequence encoding the partial amino acid sequence. The second step employed inverse-PCR which was applied to reveal the complete genomic DNA sequence encoding the protein but included introns. The primers used were designed from partial

[☆] CS designed the primers and developed the technical approach and drafted parts of the manuscript. JDM contributed to the development of the research strategy and wrote the manuscript.

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sequences and the template from self-ligated genomic DNA fragments digested by restriction enzymes. The last step used was RT-PCR which was used to get the cDNA sequence and remove introns.

The internal N-terminal sequence was obtained from Edman degradation (Sick Kids Hospital, University of Toronto). Degenerate primers of Asp v 13 were designed from both the Edman degradation sequence and the conserved domains from its homologous proteins from the internal-terminal sequence (Table A.1.). Functional degenerate primer pairs were used. These were AV41kD-M-F and AV41kD-C-R for amplifications within the Asp v 13 gene. The PCR template used was 100 ng genomic DNA extracted from *A. versicolor* mycelium (UltraClean™ Microbial DNA Isolation Kit, 12224, MO BIO Laboratories, Inc., CA). Inverse PCR with primers AVS-In-F and AVS-In-R (Table A.1.) were then applied to obtain the genomic DNA sequence encoding whole Asp v 13 protein. Finally, RT-PCR was run with total RNA as the template and AVS-N-F-Start/AVS-C-R as the primers to get its cDNA sequence and the introns were determined from the comparison of its cDNA and genomic DNA sequence.

2.2. Protein expression

Expression of recombinant Asp v 13 was done in the pET101 vector (K10101, Invitrogen, Carlsbad, CA) in the host strain BL21-CodonPlus (DE3)-RIL. This was induced using 1 mM IPTG and allowed to grow overnight at 20°C (Shi et al., 2011). The protein was localized in the soluble fraction and purified by Ni-NTA (30410, Qiagen, Mississauga, ON) according to manufacturer's instructions. The purified recombinant Asp v 13 was then characterized by 12% SDS-PAGE with SeeBlue Plus2 Pre-Stained Standard (LC5925, Invitrogen). Protein bands were visualized with GelCode Blue Stain Reagent (24592, Pierce, Rockford, IL) according to the manufacturer's instructions. The presence of recombinant Asp v 13 was then confirmed with immunoblots using monoclonal antibodies IgM raised to Avs43 but equally responsive to Asp v 13 (2H12 and 8A10), an enhanced polyclonal antibody from goat sera (Liang et al., 2011) and anti-His antibody (R93025, Invitrogen), followed by the corresponding secondary anti-mouse IgM conjugated with alkaline phosphatase (for 2H12 and 8A10), anti-goat-IgG (for polyclonal antibody) conjugated with alkaline phosphatase or, or anti-mouse-IgG (for anti-His) conjugated with alkaline phosphatase, respectively. BSA was loaded in the same gels as Asp v 13 in all the immunoblot experiments as the negative control to eliminate the possibility of unspecific binding. The secondary antibodies were detected by incubating membranes with BCIP/NBT (B3679, Sigma, Oakville, ON) for 5–10 min.

2.3. Homology analysis

Basic Local Alignment Search Tool (BLAST) searches were carried out for the fragment from Edman degradation sequences of Asp v 13 (National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov>). Protein sequences were acquired from the non-redundant SwissProt (Swiss Institute of Bioinformatics, Basel, Switzerland) as a reference database. The alignments of Asp v 13 with their respective homologous proteins were run by T-COFFEE (<http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee.cgi/index.cgi>) (Poirot et al., 2003; Notredame et al., 2000).

2.4. ELISA analysis

The ELISA analysis followed the protocol of Xu et al. (2007, 2008), with minimal modification. 100 µl (2 ng/µl) purified recombinant Asp v 13 or different concentrations of the purified recombinant

Asp v 13 from 200 ng/well to 0.195 ng/well in 50 mM pH 9.6 carbonate bicarbonate buffer (C3041, Sigma) were kept overnight at 4°C to coat the microwells of the plate. After the plate was blocked with BSA, diluted with 2% BSA/PBS solution, 100 µl atopic human sera from patients shown to be IgE positive to the fungus was added (Liang et al., 2011). Non-atopic human serum from Sigma (S2145) was used as a negative control. These were used in assays of the recombinant Asp v 13. A 1:2000 dilution of anti-Avs43 IgM 2H12 and 8A10 (Liang et al., 2011) or 1:1000 dilution of anti-Avs43 polyclonal antibody was used for the binding. After incubation and washing, 100 µl anti-human IgE conjugated with horseradish peroxidase (A9667, Sigma), anti-mouse IgM conjugated with horseradish peroxidase (A8786, Sigma) or anti-goat IgG conjugated with horseradish peroxidase (A5420, Sigma) in 1% BSA/PBST were added as the secondary antibody as appropriate. Each atopic serum, mAb and pAb assay was done triplicate in at least three separate experiments. SYSTAT v. 12 was used for statistical analysis.

2.5. Epitope mapping

Epitope mapping was done with synthesized peptides using the spot peptide technique (Frank, 2002; hereafter the SPOT method). Asp v 13 is a 403 amino acid protein. A total of 67 peptides covering the entire region of Asp v 13 were synthesized on a modified cellulose membrane (Kinexus Bioinformatics Corporation, Vancouver, BC). Each peptide was 12 amino acids long overlapping by six amino acids with six amino acids offset between two adjacent peptides. Epitope mapping was conducted using human sera from patients shown to be IgE positive to one of a variety of fungi (1:100 dilution v/v). The membrane was soaked overnight in 20 mL of Tris-buffered saline (TBS) containing 1% BSA (pH 7.5), and then washed three times with TBS containing 0.1% (v/v) Tween (TBST). A 1:10 (v/v) diluted pool of atopic patient sera or non-atopic human sera as the negative control was added and the membrane was incubated for 2 h at room temperature. After three washes with TBST, the membrane was incubated in a 1:4000 dilution of mouse monoclonal anti-human IgE-HRP or 1:4000 dilution of anti-human IgG-HRP (A0293, Sigma) for 1 h. After three washes with TBST, the interacting peptide spots were developed with ECL kit (SL100309, SigmaGen® Laboratories) for 3–5 min following its manual's instruction and imaged by the FluorChem® Q Imaging System (Cell Biosciences, Santa Clara, CA). The membrane was used a number of times after a regeneration step consisting of one wash in methanol for 1 min, three washes in deionized water, three washes in 8 M urea containing 1% (w/v) SDS, 1% (w/v) β-mercaptoethanol followed by three washes in deionized water.

2.6. Homology-Model of Asp v 13 protein

The three-dimensional structure of a subtilisin-like serine protease (1r6v, Protein Database Bank (<http://www.pdb.org/pdb/home/home.do>)) was used as the template to build the homology model of Asp v 13 three-dimensional structure. The homology model of Asp v 13 was generated using program ESyPred3D (<http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/esypred/>) (Lambert, 2002) and viewed by Swiss-PdbViewer (<http://spdbv.vital-it.ch/>). The energy minimization of Asp v 13 three-dimension model was performed *in vacuo* with GROMOS96 43B1 set without a reaction field (Schuler et al., 1996). The stereochemistry and overall quality of the homology model were checked using the software Procheck (Laskowski et al., 1993). The final model of Asp v 13 included residues 23–382.

2.7. Phylogenetic relationships

An un-rooted phylogenetic tree was built from the available amino acid sequences of Asp v 13 and other subtilisin-like serine proteases from the pubmed database. This was done using the PHYML algorithm (Dereeper et al., 2008); available from (<http://www.phylogeny.fr/version2.cgi/simple.phylogeny.cgi>). The tree was rendered with TreeDyn (Chevenet et al., 2006).

3. Results

3.1. Asp v 13 protein sequence

The internal N-terminal amino acid sequence of Asp v 13 obtained from the Edman degradation was ALTTQSDAPPGLGAISHQGDAS(A)SSYI (Liang et al., 2011). BLAST analysis indicated that it was predicted to be a fungal serine protease. Related proteins included AAA67705 from *Aspergillus nidulans* FGSC A4 (69% identity; 76% positive), ACX47962 from *Aspergillus clavatus* (69% and 73%), EAU35702 from *Aspergillus terreus* NIH2624 (72% and 72%) and AAB29384 from *Aspergillus flavus* (72% and 72%). The conserved domains from the alignment of the homologous proteins to Asp v 13 were used in the design of degenerate primers together with Asp v 13 Edman degradation sequences (Table A.1.). After sequencing of the touch-down PCR products by degenerate primers, the genomic sequence encoding 143 amino acids of Asp v 13 was identified for future inverse PCR (data not shown).

Asp v 13 Inverse PCR was then done to obtain the sequence encoding Asp v 13. Genomic DNA was separately digested with *Pst*I or *Bam*HI or *Eco*RI and the digested fragments were self-ligated as inverse PCR templates. A concentration of 2ng/μl was used for maximal self- and minimal cross-ligation. The largest inverse-PCR amplicons of Asp v 13 were then ligated to PCR2.1 and sequenced. Based on the sequences from the inverse-PCR amplicons, the genomic sequence encoding Asp v 13 from the N-terminal starting codon to the stop codon was assembled. RT-PCR was subsequently done with N-terminal forward primers and C-terminal reverse primers based on built inverse-PCR gene sequence coding Asp v 13 (Table A.1.; AVS-N-F-START and AVS-C-R). These products were sequenced to reveal the cDNA sequences of Asp v 13. Comparison of the genomic and cDNA sequences indicates that Asp v 13 contain 3 introns (Fig. 1, highlighted area). Asp v 13 was predicted to comprise 403 amino acid residues and have a molecular weight of ~42 kDa. This accords with that of the natural Asp v 13 isolated from *A. versicolor*, i.e. the protein without post-translational glycosylation of the 43 kDa protein by SDS-PAGE (Liang et al., 2011). The cDNA sequence of Asp v 13 contained the peptide sequence from Edman degradation (Fig. 1). This confirms the identity of the genomic DNA and cDNA sequences. The nucleotide and deduced amino acid sequences of Asp v 13 were deposited in Pubmed as GU827714 and ADE74975 respectively.

The sequence obtained extended into the 5' un-translated region of Asp v 13. The potential promoter of Asp v 13 was calculated as: TCGCAAAGGTATAAGAAGAGACGTGTGCCCGTCGCTCTTCCCTGACA determined by the NNPP software <http://www.fruitfly.org/seq.tools/promoter.html>; (Fig. 1, small size underlined italic) (Reese, 2001). In addition, the amino acid sequence of Asp v 13 contained 2 putative N-glycosylation sites at positions of Asn253 (Asn–Leu–Ser) and Asn291 (Asn–Thr–Ser). Both are indicated by the sequence in bold with dashed boxes in Fig. 1.

BLAST analysis of the full length Asp v 13 sequence suggested similarity to a family of subtilisin-like serine proteases from other filamentous fungi. These included but are not limited to the follows, such as the serine protease from *A. nidulans* (XP.663162,

E-value 0.0), Alp1 from *Neosartora fischeri* [related to *Aspergillus fumigatus*] NRRL 181 (XP.001266852, E-value 0.0), Alp1 from *A. fumigatus* Af293 (CAA77666, E-value 0.0), elastinolytic serine proteinase from *A. flavus* (P35211, E-value 2×10^{-171}), Alp1 from *A. clavatus* (XP.0012172038, E-value 2×10^{-165}) and a hypothetical protein from *Hypocrea virens*, the teleomorph of *Trichoderma virens* (AAO63588, E-value 6×10^{-123}). All of these fungal proteins are secretory based on sequence analysis by SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>; data not shown). This belongs to a conserved family of inhibitor.I9 superfamily (22–121) and peptidase S8 PCSK9 proteinase_K superfamily (subtilisin-like serine proteases; 122–403). The alignment tool 'T-COFFEE' was used to compare the Asp v 13 sequence with these homologous proteins (Fig. A.1). All have two conserved domains of histidine and serine active sites (Fig. 1 and Fig. A.1).

3.2. Asp v 13 overexpression and recombinant Asp v 13 characterization

The over-expressed recombinant Asp v 13 was localized in the soluble fraction (data not shown). His-tagged Asp v 13 was purified from *E. coli* by Ni-NTA column affinity chromatography and subsequently determined as a ~80 kDa dimer in SDS-PAGE gel with a yield of 1 mg/l (Fig. 2)A. Western blots verified that this over-expressed recombinant Asp v 13 dimer bound to the respective monoclonal IgM 2H12 and 8A10, polyclonal anti-Asp v 13 and anti-His antibodies respectively (Fig. 2B). BSA (negative control) did not show any bands on the same membrane, which eliminate the unspecific binding of purified recombinant Asp v 13 with these antibodies.

Recombinant Asp v 13 was strongly bound to 2H12, 8A10 and polyclonal antibody (Fig. 2C and D), further confirming the similar conformation of recombinant Asp v 13 to the natural Asp v 13 (Liang et al., 2011). Among 59 human sera tested using non-atopic human sera as the negative control, 36 sera significantly responded to recombinant Asp v 13 compared to the control (p -value < 0.001).

3.3. Asp v 13 IgE and IgG binding epitope characterization

The human sera positive to the target protein QC1294, QC1297, QC2123, QC2401 and QC2407 selected to examine the IgE-binding epitopes, six human IgE-binding peptides were identified by the SPOT method (Fig. 3B; for convenience epitopes are numbered 1–6). These are located in the amino acid sequence of Asp v 13 with highlighted and bolded letters (Fig. 3A and B). The critical amino acids for IgE binding epitopes are well-exposed in the surface of Asp v 13 molecule from the model structure (Fig. A.2) and underlined along the sequences (Fig. 3B). Of the sera tested, four were similar to the response of QC2407 and only QC2401 reacted differently. This serum strongly reacted with part of epitope #1 (13-ALLPAVFGAPILEARRQT-30) as well epitope #6 (Fig. 3A and B). These data confirm that Asp v 13 consists of a several major allergenic linear epitopes.

To identify the region of Asp v 13 that could serve as the anti-genic epitope to IgG, the IgG-binding ability of the same sera was tested for response to IgG. A total of 7 IgG-binding epitopes ca. 50% of the whole sequence were revealed (Fig. 4)B. These amino acids are also exposed on the surface of Asp v 13 molecule based on the model (Fig. 4B and Fig. A.2). The italicized amino acids in peptide #1 and peptide # 7 do not appear in the 3D structure model because of the truncated modeling. These results also confirm Asp v 13 consists with major antigenic epitopes binding with atopic human sera IgG.

Asp v 13 was then compared by alignment for the similarity with Asp f1 1 from *A. flavus*. LALIGN program showed the whole sequence of Asp v 13 has 73.6% identity and 92.8% similarity with Asp f1 1

TGAATCCTTGTCATTGCGGATGAGGGGATGAATGACGCGAGGCATAGATATATTGGAGTGC **TCGCAAAAGGTATAAGAAGAGAGCGTGTGCCCGTCGCTCT** -89
 Promoter
TTCCCTGACAATCAGCAAAGCAAACCTCTCTCTTTCTGAAACAATCTCATCCAGCTACGTTTCTGGGTTTTCTCAGAAATCCCATC 0
ATGATTCAATCAAGCGCAGCATCTCTCTCTCGGAGCTCTTCTTCGCGCTCTTGGTGCTCTATCCTCGAAGCT 78
 1 M Y S I K R T I L L L G A L L P A V F G A P I L E A
 CGCCGGCAGACAGAAAAGGTCCCGGGGAAGTACATCGTGACGTTCAAATCTGGCCTGCAGGCCGAGCAGATCGAC 153
 27 R R Q T E K V P G K Y I V T F K S G L Q P E Q I D
 GCACACAACTTGGGCCTCGAAGCTCCACAAGCGCAACCTAGAGCGCCGCGATCTGACTGACCGTGATTGTAC 228
 52 A H T T W A S N V H K R R N L E R R D L T D R D L Y
 CCCGGTATTGAGAAGAACTTCAAGATCCACAATTCGCGCGTACGTTGGTCTTTTGATGATGCCACCATCGAAGAA 306
 77 P G I E K N F K I H K F A A Y V G S F D D A T I E E
 ATTGAAACCACAAAGAT **GTCTGGTGCCCGCTCTTGAATTC**CCCCAGTTGAAGCTATAATTAATTTGGACCGATCCC 384
 103 I R N H K D
CCCCAGTGGCTCACGTGGAAGAGGACCAAGTCTGGTACCTTGATGCCCTAACCCACCCAGAGCGACGCTCCCTGG 459
 109 V A H V E E D Q V W Y L D A L T T Q S D A P W
 GGTCTTGGGGCTATCTCTACCAAGGGGATGCGAGCTCCGACTATATTTACGACACAAGCGCTGGAGCTGACACT 534
 132 G L G A I S H Q G D A S S D Y I Y D T S A G A D T
 TACGCATACGTGGTGGACACCGGCATCAATGTTGATCACTCGGAATTTGACGGACGCGCCAGTCTGGCCTACAAC 609
 157 Y A Y V V D T G I N V D H S E F D G R A S L A Y N
 GCTGCTGGTGGCCAGCAGTTGACAGCGTTGGCCACGGTACCCATGTTGCTGGCACTATCGGTGGCAAAACCTTC 684
 182 A A G G Q H V D S V G H G T H V A G T I G G K T F
 Histidine active site
 GGTGTTTCTAAGAAGGCCAATCTTCTCTGTCAAGGTCTTCGAGGGCGAATCTAGCAGCACCTCCATCATCCTCGAC 762
 207 G V S K K A N L L S V K A F E G E S S T S I L D
 GGCTCAACTGGGCTGTAACGACATCGTAAGCAAGACCGCGACCGGCAAGTCGGCAATCAACCTGAGCTTGGGTA 838
 233 G Y N W A A N D I V S K S R T G K S A I **N L S** L
AGTATTCGCTACTGCCTTCCCGAGGGGGGTAAACACTGCTGACTGGACATTGTAGGCGGTGGATACTCTTATGCC 913
 257 G G G Y S Y A
 TTCAGCAACGCAGTCGAAAGCGCGTTTGATGAGGGAGTCTCTCCGTTGTAGCAGCTGGTAACGAGAAC **GTGAGTCT** 990
 264 F S N A V E S A F D E G V L S V V A A G N E N
ATTCAATATTATCTACTCCAAAACCGATGTCTAACAAGCGTGCAACCTCAGGTTGATGCATCGAACACCAGTCCCGCT 1068
 287 V D A S **N L S** P A
 TCCGCACCTAACGCTCTTACTGTTGCGGCTTCCACGGAAAGAAATGCCCGCGCATCGTTCTCCAACCTACGGTGAA 1143
 296 S A P N A L T V A A S T E R N A R A S F S N Y G E
 GTGGTCGACATCTTTGCCCTGGTGAAGACATCTCTCGGCCTGGATTGGCGGAAACAGTGGCACTAACACCAATT 1218
 321 V V D I F A P G E D I L S A W I G G N S A T N T I
 TCTGGCACATCTATGGCCACTCCCATATTGTTGGATTGTCCCTCTACTTGATCGCTCTCGAGGGCCTCTCTCTCCC 1296
 346 S **G T S M A T P H I V G** L S L Y L I A L E G L S S P
 Serine active site
 GGGGATGTGACTTCCCGAATCAAGGAGCTGGCTACCCAGGGCGCTCTCTCGGGCGTGTCTGGCAGCCCCAATGCG 1371
 372 G D V T S R I K E L A T Q G A L S G V S G S P N A
 CTTGCTTACAACGGCGCTGAA **AA**ACAGCTAGCTCGAAGCTGATTGCAATAAGTCCCGTGGTCTGGTGAATCAAGAGATCAAGATATA 1467
 397 L A Y N G A E —
 AACGCAATGCAGAAATAGCAAGAGCCCTGACGGCTTGATAAGTCGTTACGAATGCACATATTTTATGGTCAATACATAGTTCTCATTACGTAGATCTGCCGAC 1571
 GATCCAGCAGCAAAATCCTGAAGAAAGTAAATTTGATCTTCGTCCATCTCAGTCTGGGTAGTCTCGGGC 1643

Fig. 1. Nucleotide sequence of *A. versicolor* Asp v 13 gene, its bordering regions and amino acid sequence deduced from Asp v 13 gene. Translation initiation codon ATG was positioned at nt +1 in right and first Met was positioned 1 in the left. Translation terminator codon TAA solidly boxed was located at nt +1393. The speculated promoter region was italic and underlined. The three intron sequences were highlighted in grey. The conserved amino acids for Histidine active site and Serine active site were bold and underlined. The amino acid sequence for potential N-linked glycosylation site was boxed with dashed line.

(data not shown). Each of the IgE and IgG binding epitopes were similar to the homologous fragments of Asp f1 1 (Yu et al., 1999). The IgE-binding epitopes from #1 to #5 shared their highest identity of 66.7%, 64.7%, 75.0%, 66.7% and 83.3% and highest similarity of 91.7%, 88.2%, 100.0%, 100.0% and 100.0% with Asp f1 1 (Q9UVU3) respectively (Yu et al., 1999; Chou et al., 1999). While the IgE binding epitope #6 shared their highest identity of 63.6% and highest similarity of 90.9% with Tri m 2, an allergen from *Arthroderma benhamiae* (CAD23613.1; data not shown). All the IgG binding epitopes from #1 to #7 share their highest identity of 72.7%, 64.7%, 70.8%, 83.3%, 71.4%, 79.2% and 70.2% and highest similarity of 95.5%, 88.2%, 100.0%, 100.0%, 88.1%, 100% and 91.5% respectively with Asp f1 1 (Yu et al., 1999) (Fig. 5).

4. Discussion

There are more than 20 allergens that have been identified from *A. versicolor* including glyceraldehyde-3-phosphate dehydrogenase, sorbitol/xylulose reductase, catalase, enolase, malate

dehydrogenase and other hypothetical proteins (Benndorf et al., 2008). We detected three proteins with the molecular weights of 68, 43/41 and 30 kDa that were antigenic. Of these the 43 kDa and 41 kDa proteins were most consistently recognized by reactive sera and most widely distributed in the strains examined. In the present study, PCR analysis using a degenerate primer, inverse PCR and RT-PCR revealed that the 43/41 kDa protein (Liang et al., 2011) is comprised of 403 amino acids (Fig. 1). It was ultimately given the IUIS designation Asp v 13. Approximately 1803 bases coding Asp v 13 were identified with 3 introns inside the open read frame. The molecular weight of the complete protein was 42.5 kDa with calculated pI of 5.2 (<http://www.scripps.edu/cgi-bin/cdputnam/protcalc3>). Signal peptide analyses of Asp v 13 and its homologous proteins by SignalP 3.0 showed Asp v 13 contained a 21 amino acid signal peptide MYSIKRTILLGALLPAVFGA (0.999). This is consistent to our previous report that Asp v 13 is an extracellular protein (Liang et al., 2011). The mature molecular weight of Asp v 13 without the signal peptide was 40.2 kDa with calculated pI of 5.05. This is similar to the measured pI of

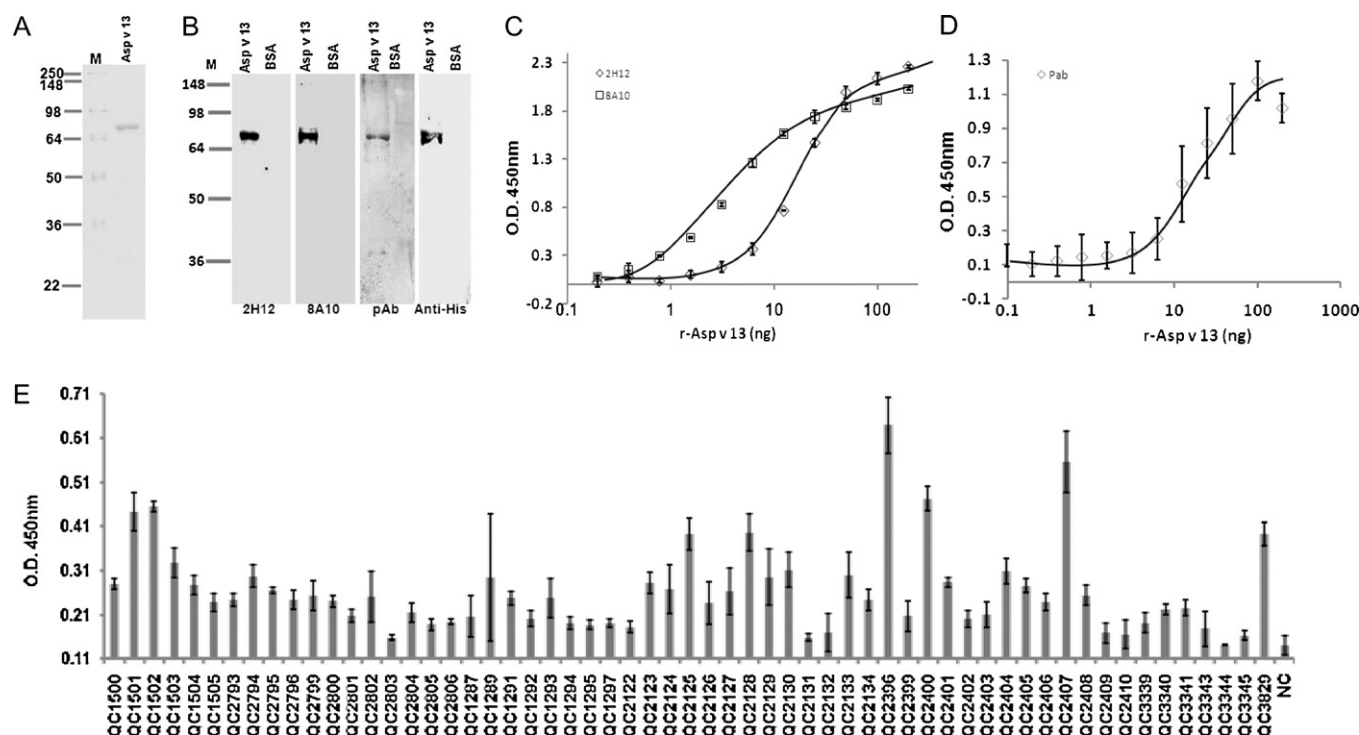


Fig. 2. SDS-PAGE, Immunoblot and ELISA analysis of recombinant Asp v 13 over-expressed in *E. coli* cells. Panel A shows the Coomassie Brilliant Blue-stained gel of Asp v 13 over-expressed in DE3-RIL host cells and purified by Ni-NTA. Panel B is the western blot of purified recombinant Asp v 13 with monoclonal 2H12 and 8A10, Pab and anti-His as the primary antibody and anti-mouse IgM (For Mab) and anti-mouse IgG as the secondary antibody. The control lanes are the negative controls by loading BSA. Values at left in panel A and B indicate molecular weights in kDa. Panels C and D are the indirect ELISA by using Mab 2H12 and 8A10 (C) and Pab (D) as the primary to detect the immuno-interaction with rAsp v 13 respectively. Panel E shows binding to rAsp v 13 by IgE from each of four atopic individuals human sera labeled across top, NC in the right side is the negative control by non-atopic human sera.

the native protein (4.5; Liang et al., 2011). The difference is most likely explained by the proteins with, and without, the signal peptide. However, differences resulting from post translations cannot be excluded.

Serine dependent proteases are widespread and have been divided to six groups. Subtilisin-like serine proteases is one of the largest of these with a protein scaffold α/β and Asp/His/Ser of catalytic residues in the polypeptide chain (Barrett

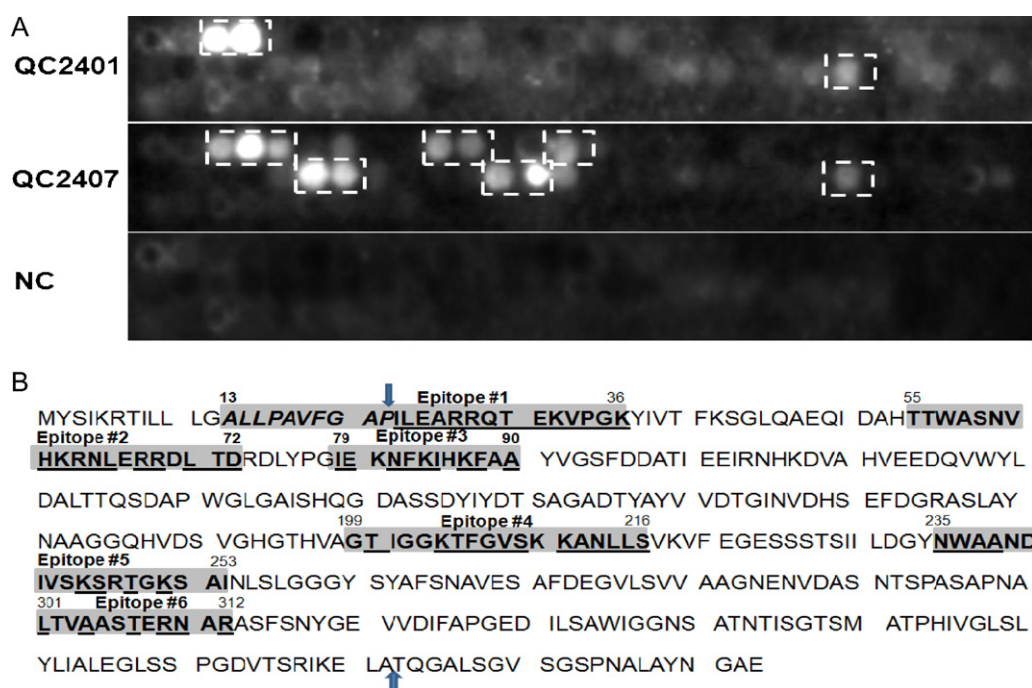


Fig. 3. IgE binding epitopes of Asp v 13. Panel A, examples of Spot results were obtained with Asp v 13 using human sera and anti-human IgE as a probe. Non-atopic human serum (NC) was included as the negative control. Panel B, IgE-binding epitopes deduced from the stained spots are highlighted with bold letters along the amino acid sequences of Asp v 13. Wholly exposed amino acids along each epitopes were underlined. The arrows show the beginning and the end of Asp v 13 model.

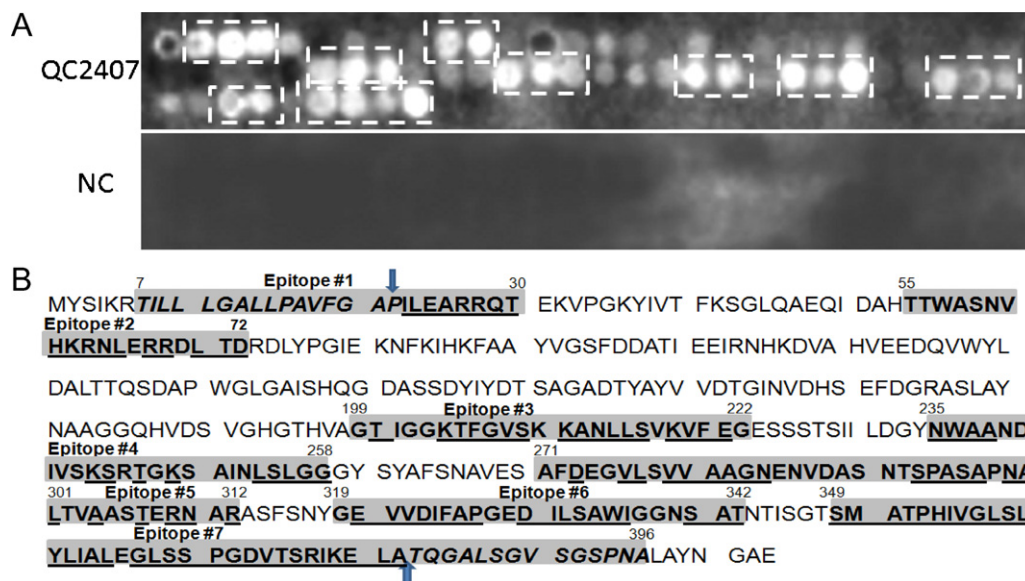


Fig. 4. IgG binding epitopes of Asp v 13. Panel A, examples of Spot results were obtained with Asp v 13 using human sera and anti-human IgG as a probe. Non-atopic human serum (NC) was included as the negative control. Panel B, IgG-binding epitopes deduced from the stained spots are highlighted with bold letters along the amino acid sequences of Asp v 13. Wholly exposed amino acids along each epitopes were underlined.

and Rawlings, 1995). The three-dimensional model of Asp v 13 had seventeen β -strands and seven α -helices (Fig. A.2). BLAST searches for conserved domains and homologous proteins (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and a phylogenic tree calculation (Fig. A.3) confirmed Asp v 13 as a subtilisin-like serine protease. This novel serine protease comprised three main domains: the pre-sequence signal peptide in the N-terminus from M1-A21; the pro-sequence domain from P22 to D121 for correct folding and self inhibition (Yabuta et al., 2001), and the domain from A122 to E403, as the functional protease. This super family has highly conserved Asp, Ser and His active sites (Fig. 1 and Fig. A.1). The pre-sequence signal peptide and pro-sequence domain from P22-D121 can be removed by specific protease and auto-proteolysis respectively (Yabuta et al., 2001). Subtilisin-like serine protease is widely distributed in pathogenic fungi (Huang et al., 2004). Subtilisin-like serine proteases have been identified

as allergens of *Penicillium*, *Aspergillus* and *Trichophyton* species (Yu et al., 1999; Chou et al., 1999, 2002; Tai et al., 2006; Shen et al., 1999, 2007; Pöll et al., 2009). Clinical tests suggested that Pen ch 1, a subtilisin-like serine protease from *Penicillium chrysogenum* induced the release of histamine from peripheral blood leucocytes of asthmatic patients (Chou et al., 2002). In a murine model, subtilisin-like serine protease from *A. fumigatus* resulted in lung inflammation (Kurup et al., 2002).

Recombinant Asp v 13 showed similar characteristics to the native protein. A strong affinity was seen for a number of monoclonal antibodies prepared from the native protein as well as an enhanced polyclonal antibody (Fig. 2B, C and D). Native 41/43 kDa protein (Liang et al., 2011) reacted to 20% of the human sera randomly selected from our human sera pool by using immunoblot. Recombinant Asp v 13 had high response to 23 of 59 selected human sera by using indirect ELISA (Fig. 2E). This indicates that the con-

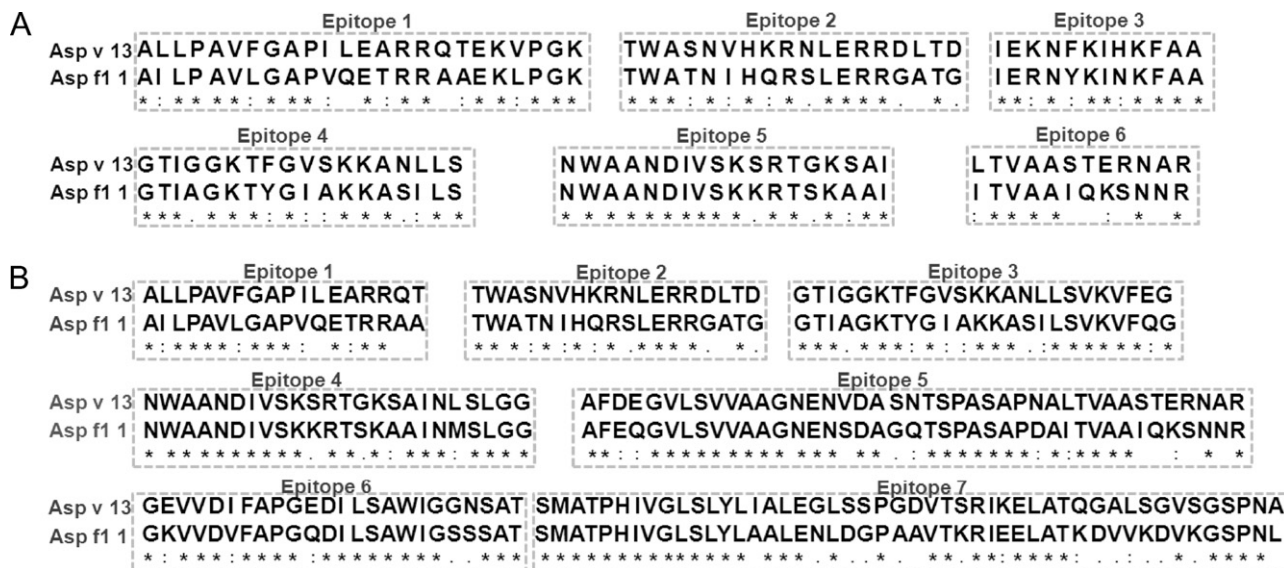


Fig. 5. Alignment of Asp v 13 with allergen Asp f1 1. Panel A, IgE binding epitopes of Asp v 13 were aligned with their homologous fragments from Asp f1 1. Panel B, IgG binding epitopes of Asp v 13 were aligned with their homologous fragments from Asp f1 1. Identical (*), highly similar (:), and similar (.) amino acids across all sequences are indicated below alignment.

formation between recombinant and native Asp v 13 is similar. However the recombinant Asp v 13 is apparently a dimer (Fig. 2A) while natural Asp v 13 is a monomer on SDS-PAGE (Liang et al., 2011).

The incomplete three-dimensional model (residues 23–382) for Asp v 13 was built using the template structure of 1r6v (a subtilisin related keratinolytic enzyme from *Fervidobacterium pennivorans*), which shares 31% similarity with Asp v 13 by using LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html). The quality of its three-dimensional model was evaluated by PROCHECK and the most favored regions of Ramachandran Plot statistics was 82.2% comparing to 86.7% of the template structure of 1r6v from X-ray diffraction with 1.7 Å resolution (Kim et al., 2004), suggesting the good quality of Asp v 13 three-dimension model structure for the epitopes localization.

The Asp v 13 three-dimensional model comprises two domains (A and B) with 17 β-strands and seven α-helices (Figs. A.2A and A.2B). These two domains are conserved to Inhibitor.I9 super family (23–121) and peptidase.S8.PCSK9.Proteinase.K super family (122–382) respectively, both of which adopt globular shapes separately exhibiting a predominantly negatively charged from in the view of both the left and right sides (Figs. A.2G and A.2H). Two putative N-glycosylation sites occur at positions Asn253 (Asn–Leu–Ser) and Asn291 (Asn–Thr–Ser) but, according to the GlyProt server for glycosylation analysis (<http://www.glycosciences.de/modeling/glyprot/php/main.php>), none of both sites are actually glycosylated due to their unfavorable geometric conditions and both Asn253 and Asn291 were buried (Fig. A.2). This is consistent to our previous report that natural Asp v 13 was not a glyco-protein (Liang et al., 2011).

Many subtilisin-like proteases from different fungal species have been studied for their allergenicity (Yu et al., 1999; Chou et al., 1999, 2002, 2003; Tai et al., 2006; Shen et al., 1999; Shen et al., 2007; Su et al., 1999; Chow et al., 2000). As one of the most prevalent allergens, the epitopes of the subtilisin-like serine protease allergens, such as Tri r2 (Gao and Takashima, 2004), Pen n 18 (Yu et al., 2002), Pen ch 18 (Shen et al., 2007) and Cla h 9 (Pöll et al., 2009) have been widely probed. In this report, a total of six IgE binding epitopes and seven IgG binding epitopes of Asp v 13 were characterized along the amino acid sequences. This was done by systematically probing the antigenic activity of sequence-derived SPOT peptides with atopic human sera (Figs. 3 and 4). All these IgE and IgG binding epitopes contain charged residues. In addition, all the IgE and IgG binding epitopes are mostly exposed in the Asp v 13 molecular surface from its three-dimension model (Fig. A.2), suggesting their possible strongly reorganization by IgE and IgG on the molecular surface of Asp v 13. The conservation of both IgE and IgG binding epitopes with the allergens such as Asp f1 1 (Yu et al., 1999) and Tri m 2 (CAD23613.1) (Fig. A.1 and Fig. 5) and that both natural and recombinant Asp v 13 react to the atopic human sera (2; Fig. 2E) implies some cross-reaction of Asp v 13 with other homologous allergens especially from *Aspergillus spp.*, *Trichophyton spp.* and *Penicillium spp.*

Although the SPOT technique used here only maps linear IgE and IgG binding epitopes, the finding that IgG and IgE binding epitopes of Asp v 13 strongly reacted with atopic human sera is the first stage to design a recombinant allergen to improve the diagnosis of *A. versicolor* allergy in the clinic and, potentially, immunotherapy (Beyer, 2003; Zeiler and Virtanen, 2008; Chapman, 1991).

Conflict of interest

The authors have no conflicting financial interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2011.05.010.

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Table A.1. The primer names, sequences and purposes in this study.

Primers	Sequences in 5' to 3' end orientation	Purpose
AV41kD-M-F	GGC CAY GGH ACB CAYGTT GCH GGH ACH ATT GGY GGC DMD ACC TAC GGH G	Fragment Sequence
AV41kD-C-R	CCR ACA AYG TGR GGD GTV GCC ATG GAR GTR CCR SWG ATB GTG TTG GTR GC	Fragment Sequence
AVS-In-F	GTT GAT GCA TCG AAC ACC AGT CCC	Inverse PCR
AVS-In-R	GCG GCT CTT GTC TAC GAT GTC G	Inverse PCR
AVS-N-F-Start	ATG TAT TCA ATC AAG CGC ACG ATC CTC C	cDNA Synthesis
AVS-C-R	TTC AGC GCC GTT GTA AGC AAG CGC	cDNA Synthesis & pET101 cloning
AVS0-F	CACC ATG TAT TCA ATC AAG CGC ACG ATC CTC C	pET101 Cloning

Fig. A.1.

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Avs41      MYSIKRT--ILL---LGALLP-AVF-----GAPILE--ARRQTEKVPKGYIVTFKS
P35211.1   MQSIKRT--LLL---LGAVLP-AVL-----AGPIFP--HRRAPTTIPGKYIVTFKS
XP_001266852.1 MLSIKRT--LLL---LGAVLP-AVF-----GAPVQE--TRRAAQKIPGKYIVTFKP
CAA77666.1 MLSIKRT--LLL---LGAVLP-AVF-----GAPVQE--TRRAAQKIPGKYIVTFKP
AAO63588.1 MASIRRL--ALY---LGALLP-AVL-----AAPAG--HHKAPAEVFNKIVTLKE
CAL25580.1 MASIRRL--ALY---LGALLP-AVL-----AAPAA--HHKAPAEVFNKIVTLKE
CAD24290.1 MLF-KSI--IAA---VSAFAG-LSA-----AAPA--NIRASNVIEGSIYVKLKD
AAP15044.1 MTSIRRL--ALY---LGALLP-AVL-----AAPAA--LHKKEAPAFNKIVTLKE
ABV59216.1 MGTVSSRRSWP--L-PLLLLLLLLPGAGARAQDEDEGDYEEVLALRSEEDGLAEAPHGTTATFHR--CAKDPWRLPGTYVVVLKE
AAK54902.1 MISNGLT--SL--L-AIAG-LAF-----AGPIRKVSNAGASGTADKYIVVLKN
MGTCHSANTLWRLPLPLPLLLLLLLLCTGAGAQDE--DGDYEEIMLALPSQEDGLADEAAHVATATFRR--CSKEAWRLPGTYVVVLME
AAA87324.1 MNKMKMK--IVA---GTALIIISVAF--S-----SS--IAQAAEEAKEKYLIGFKE
MGIRCSTWLRLWP--L-SPQLLLLLLLCPTGSRAQDE--DGDYEEIMLALPSQEDSLVDEASHVATATFRR--CSKEAWRLPGTYVVVLME
AAU14826.1 MKRTWYL--VGL---FSALLGACQ--M---SSKNPTPT-----LAEAFWPK-EAPVY--GLDDPEAIPGRYIVVFKE
*
Avs41      GLQAEQIDKHTWASNVHK---RNLERRDLTDRLDYPGIEKNFKIHKFAAYVGSFDDATIEIRNHKDVHAEVEDQVWYLD-----AL
P35211.1   DVDQAADIKHATWATDIHK---RNLRQRRSSEEDLPIGIERNFKINKFAAYSGSFDEDTIAQIRQSDVEAAVEDQVWHLF-----DL
XP_001266852.1 GTDAATIESHTLWATDLHK---RNLERRDATSGEPPIGIEKNYKIKDFAAYAGSFDDATIEIRKSDADVHAEVEDQIWIYD-----AL
CAA77666.1 GTDTATIESHTLWATDLHK---RNLERRDTSGEPPVIGIEKSYKIKDFAAYAGSFDDATIEIRKSDADVHAEVEDQIWIYD-----AL
AAO63588.1 GASID--TDSHLTWNDLHR---RSLTKRNT---AGVEKTYNIHTWSAYAGEFDEDTIEQIKASPDVASVEPDYIMYLSDIVEDKRAL
CAL25580.1 GASVD--TDSHLTWNDLHR---RSLTKRNT---AGVEKTYNIHTWSAYAGEFDEDTIEQIKASPDVASVEPDYIMYLSDIVEDKRAL
CAD24290.1 NVAA--DEHISWVSIGIA---RRNVDDF---AGIQHEYNSPAIFRGYAGHFDQQTIEQIKASPDVASVEPDYIMYLSDIVEDKRAL
AAP15044.1 GASID--TSLAWNDLHR---RSLTKRST---AGVEKTYNIHTWSAYAGEFDEDTIEQIKASPDVASVEPDYIMYLSDIVEDKRAL
ABV59216.1 ETHLSQSERTA---RR---LQAQA---AR-RG-YLTKLHVF-HGLPLGFLVKMSDDLGLALKLPHVDYIEDSSVF-----
AAK54902.1 GLSESVAKHTRNRSFHSVARDLTGARA---HGVGRKFRFAATGNGYVGGFKATLQEIINSPEVDYVQDVAVKI-----N
CAM15751.1 ETQRQLQEQT---RR---LQTRA---AR-RG-YVVKLVHIF-YDLFPGLVKMSDDLGLALKLPHVEYIEDSSVF-----
AAA87324.1 QEVMSQFVDQI---DGD-EYSISS---QA---EDVEIDLHEFDIPLVSLVELDPEVDALDPAIAYIEDAE-----V
AAW31850.1 ETQRQLQEQT---RR---LQTRA---AR-RG-YVVKLVHVF-YDLFPGLVKMSDDLGLALKLPHVEYIEDSLVF-----
AAU14826.1 GKQSLQGGI---TT---LQARL---AP---QGVAVTQAYT-GALRGFAAVMAPALEVLQDDPVEFIADKAVRA-----W
*
Avs41      TTQSDAPWGLGAISHQGA--SSDYIYD--TSAGADTYAYVVDGTGINVDHSEFD---GRA SLA--YNA-----GGQHVDSVGHGTHV
P35211.1   TTQSDAPWGLGSIHSHKQP--STDYIYD--TNGGEGTYAYVVDGTGINVDHEFE---GRA SLA--YNA-----GGQHVDSVGHGTHV
XP_001266852.1 TTQKGA PWGLGSIHSHKQA--STDYIYD--TSAGAGTYAYVVDGTGINVNHVEFE---GRA SLA--YNA-----GGSHVDSVGHGTHV
CAA77666.1 TTQKGA PWGLGSIHSHKQA--STDYIYD--TSAGAGTYAYVVDGTGINVNHVEFE---GRA SLA--YNA-----GGSHVDSVGHGTHV
AAO63588.1 TTQKGA PWGLGTSHRTSG--STSYIYD--TSAGSGTYAYVVDGSGINTSHQQFG---GRA SLG--YNA-----GGQHVDTLGHGTHV
CAL25580.1 TTQKGA PWGLGTSHRTSG--STSYIYD--TSAGSGTYAYVVDGSGINTSHQQFG---GRA TLG--YNA-----GGQHVDTLGHGTHV
CAD24290.1 TTQSDAPWGLGSIHSHREAN--AAGYIHD--TAAGAGTGYVVDGSGVTRTHQEF---GRA STG--WTGY---PGDESDTLGHGTHV
AAP15044.1 TTQSDAPWGLGTSHRTSG--STSYIYD--SSAGSGTYAYVVDGSGINTSHQQFG---GRA SLG--YNA-----GGQHVDTLGHGTHV
ABV59216.1 --AQSIIPWNLERITPPRYA--DEY-Q--PPDGGSLVEVYLDTSIQSHREIE---GRVMTDFENVEEDGTRFHRQAKSDCHSGHTL
AAK54902.1 AEQLDSTWGLDRISHENFAAPTYEYDETAAGAGTYVYVDTGVQISHDEFKTANGTSRA SWG--KNFV-----DSSDSGNGCHGTHC
CAM15751.1 --AQSIIPWNLERITPAWHQT--EED-R--SPDGGSQVEVYLDTSIQGAHREIE---GRVTITDFNSVEEDGTRFHRQAKSDCHSGHTL
AAA87324.1 TMTQTPWNLINRVQAPIAQ--SRGF---TGTGVRVAVLDTGIS--NHADLR---IRG GAS--FVPG---EPNISDNGCHGTVQ
AAW31850.1 --AQSIIPWNLERITPAWHQT--EED-S--SPDGGSQVEVYLDTSIQSGHREIE---GRVTITDFNSVEEDGTRFHRQAKSDCHSGHTL
AAU14826.1 ATQSPAPWGLDRIQDRDLPLNSYTY--TATGRGVNVYVDTGIRTHQEFQ---GRA RVG--YDAF-----GGNGQDCNCHGTHV
*
Avs41      AGTIGGK---TFGVSKKANLLSVKVFEG--ESSSTSILDGYNWAANDIVSKSRGTGKSAINLSLGGGY--SYAFNSNAVESAFDEGLSVV
P35211.1   SGTIGGK---TYGVAKKANLLSVKVFEG--ESSSTSILDGFNWAANDIVSKKRTGKAAINMSLGGGY--SKAFNDAVENAFNEGLSVI
XP_001266852.1 AGTIGGK---TYGVAKKTNLLSVKVFQ--ESSSTSILDGFNWAANDIVSKGRTRKAAINMSLGGGY--SYAFNSNAVENAFDEGLSVV
CAA77666.1 AGTIGGK---TYGVAKKTNLLSVKVFQ--ESSSTSILDGFNWAANDIVSKGRTRKAAINMSLGGGY--SYAFNSNAVENAFDEGLSVV
AAO63588.1 SGTIGGK---TYGVAKQASLISVKVFQ--NSASTSVILDGYNWAANDIVSRNRASKSAINMSLGGGA--SSAWTTAINAFAFNQGVLTIV
CAL25580.1 SGTIGGK---TYGVAKQANLISVKVFQ--DSASTSVILDGYNWAANDIVSKNRASKSAINMSLGGGA--SSTWTTAINAFAFNQGVLTIV
CAD24290.1 AGTVAKKATII SVKVFQ--KEGSTSVILAGFDWAVNDIISKGRDRKSAINLSAGPY--SRANDIAEAAFTRGVLSVI
AAP15044.1 SGTIGGK---TYGVAKQASLISVKVFQ--ESATTSVILDGYNWAANDIVSRNRASKSAINMSLGGGA--SSTWTTAINAFAFNQGVLTIV
ABV59216.1 AGVVSGR---DAGVAKGASMRSLRVLNLCQKGTVSGTLIGLEFIRKSQVLIQ--PVGFLVLLPLAGGY--SRVLAACQRLARAGVVLTV
AAK54902.1 AGTIGGK---TYGVSKKAKIVAVKVLGASGSGSTSGVISMNWAQNAV---PNFVSASMSLGGK--STAINSAVDIAFNAGTTIVV
CAM15751.1 AGVVSGR---DAGVAKGTSLSHLSRVLNLCQKGTVSGTLIGLEFIRKSQVLIQ--PSGFLVLLPLAGGY--SRVLAACQRLARAGVVLTV
AAA87324.1 AGTIAAANNSIGVLGAPNVDLYGVKVLGASGSGSISGLAQGLQWANN---GMHIANNSLGSAGSATMEQAVQATASGVVLVA
AAW31850.1 AGVVSGR---DAGVAKGTSLSHLSRVLNLCQKGTVSGTLIGLEFIRKSQVLIQ--PSGFLVLLPLAGGY--SRVLAACQRLARAGVVLTV
AAU14826.1 AGTIGGV---TYGVAKAANLYAVRVLDCNGSGSTSGVIAGVDWVTQNH---RKPAVANMSLGGGA--STALDNVANSIAGGVVTV
*
Avs41      AAGNENV---DASNTSPASAPNALTVAASNENARA---SFSNYGEVVDIFAPGE DILSAWIGSNATNTISGTSMATPHIVGLS
P35211.1   AAGNENT---DASRTSPASAPDAFTVAAINVMNTRA---YFSNYGSVVDIFAPGQNILSAWIGSNATNTISGTSMATPHIVGLS
XP_001266852.1 AAGNENT---DASNTSPASAPNALTVAAINRNSARA---SFSNYGSVVDIFAPGQDILSAWIGSNATNTISGTSMATPHIVGLS
CAA77666.1 AAGNENS---DASNTSPASAPNALTVAAINKSNARA---SFSNYGSVVDIFAPGQDILSAWIGSNATNTISGTSMATPHIVGLS
AAO63588.1 AAGNGDSLGNPQVSGTSPANVPNAITVAALDINWRTA---SFTNYGAGVDVFAPGVNILLSWIGSNATNTISGTSMATPHVVGLA
CAL25580.1 AAGNGDSFGNPQVSGTSPANVPNAITVAALDINWRTA---SFTNYGAGVDVFAPGVNILLSWIGSNATNTISGTSMATPHVVGLA
CAD24290.1 DDGNAQR---DASEVSPASAPNAVITGAVDKNWRIVTNWPNQGSNGYGPVLIDFAPGDIGIESAPNSDSQTLRSGTSMASPHVAGLA
AAP15044.1 AAGNGDSFGNPQVSGTSPANVPNAITVAALDINWRTA---SFTNYGAGVDVFAPGVNILLSWIGSNATNTISGTSMATPHVVGLA
ABV59216.1 AAGNFRD---DACLYSPASAPNAVITVGATNAQDQPVTL--GTLGTNFGRCVDLFAPGE DIIAGSSDCSTCFVSQSGTSQAAAHVAGIA
AAK54902.1 AAGNESQ---DAKNVSPASAPNAVITGAIDSKNAIA---SFSNWGTGIDVFAPGV SVLSSWKSQSDTATNTISGTSMACPHVAGIA
CAM15751.1 AAGNFRD---DACLYSPASAPNAVITVGATNAQDQPVTL--GTLGTNFGRCVDLFAPGK DIIAGSSDCSTCFMSQSGTSQAAAHVAGIV
AAA87324.1 ASNGSGA---G-NVGFPARYANAMAVGATDQNNRA---TFSQYAGLIDVAPGVGVQSTVPNGY--ASFNGTSMATPHVAGIA
AAW31850.1 AAGNFRD---DACLYSPASAPNAVITVGATNAQDQPVTL--GTLGTNFGRCVDLFAPGK DIIAGSSDCSTCFMSQSGTSQAAAHVAGIV
AAU14826.1 AAGNDNA---NACNYSAPVAEALTVGATTSSTRA---SFSNYGSCVDLFAPGA SIPSAWTSDTATQTLSGTSMATPHVAGIA
*
Avs41      LYLIALEGLSPGDVTS---RIKELATQGLSG-
P35211.1   IYLSLEVLSSPKAVSD---RIKELATRGVVS-
XP_001266852.1 VYLMGLESLSGPAVTS---RIKQLATNGVVTN-
CAA77666.1 VYLMGLESLSGPAVTA---RIKELATNGVVTN-
AAO63588.1 LYLSLEGLATPVATVNSPNRIKALATSGRVTS
CAL25580.1 LYLSLEGLSTPAVATN---RIKALATSGRVTS
CAD24290.1 LYAMSVGVRGAQAVTD---HLIKNSGKGVVTP
AAP15044.1 LYLSLEGLTPTVATN---RIKALATAGRTGS
ABV59216.1 AMMLSAPFELTLAELRQ---RLIHFSAKDVINEA
AAK54902.1 AYFISAGAGDAPASITA---KIVSGAVTGQVTGN
CAM15751.1 ARMLSREPTLTTLAELRQ---RLIHFSTKDVINMA
AAA87324.1 ALVKQKNPSMNVQTRN---HLKNTATNLGNTT-
AAW31850.1 AMMLNRDPALTTLAELRQ---RLILFSTKDVINMA
AAU14826.1 ALYLBQNPSTPAVAS---AILNGATTGRLSGI

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Fig. A.1. Amino acid sequence alignment of Asp v 13 (first line) with the sequences of homologous proteins belonging to a subfamily of subtilisin-like serine protease. Identical (*), highly similar (:), and similar (.) amino acids across all sequences are indicated below alignment. The boxed squares are their conserved active sites of Histidine active site (Dashed) and Serine active site respectively. Gene Bank accession numbers of the homologous proteins are shown at left of each sequence and are from the following species (top to bottom): *Aspergillus versicolor* (Avs41), *Aspergillus flavus*, *Neosartorya fischeri*, *Aspergillus fumigates*, *Hypocrea virens*, *Hypocrea Lixii*, *Metarhizium anisopliae*, *Trichoderma hamatum*, *Homo sapiens*, *Monacrosporium elegans*, *Mus musculus*, *Bacillus subtilis*, *Rattus norvegicus*, *Thermus sp.*

B



Fig. A.2. Mapping of the immunogenic IgE/IgG binding epitopes of Asp v 13 . (A and B) Ribbon diagram of Asp v 13 showing the overall organization of α -helices ($\alpha 1$ – $\alpha 7$) and the bundle of β -strands ($\beta 1$ – $\beta 17$) from the view of right and left sides respectively. (C and D) Localization of exposed IgE-binding epitopic regions on the ribbon diagram of Asp v 13 . Epitopes are numbered and colored green (#1), blue (#2), yellow (#3), red (#4), teal (#5), and orange (#6), respectively. (E and F) IgE-binding-epitopes identified along the amino acid sequence exposed on the molecular surface of Asp v 13 . Epitopes are numbered and colored green (#1), blue (#2), yellow (#3), red (#4), teal (#5), and orange (#6), respectively. (G and H) Mapping of the electrostatic potentials on the molecular surface of Asp v 13 . Electropositively and electronegatively charged regions are colored blue and red, respectively; neutral regions are colored white. (I and J) Localization of exposed IgG-binding epitopic regions on the ribbon diagram of Asp v 13 . Epitopes are numbered and colored green (#1), blue (#2), yellow (#3), red (#4), teal (#5), orange (#6) and purple (#7), respectively. (K and L) IgG-binding-epitopes identified along the amino acid sequence exposed on the molecular surface of Asp v 13 . Epitopes are numbered and colored green (#1), blue (#2), yellow (#3), red (#4), teal (#5), orange (#6) and purple (#7), respectively. A, C, E, G, I and K were all oriented from the right side, while B, D, F, H, J and L were all oriented from the left side.

Fig. A.3.

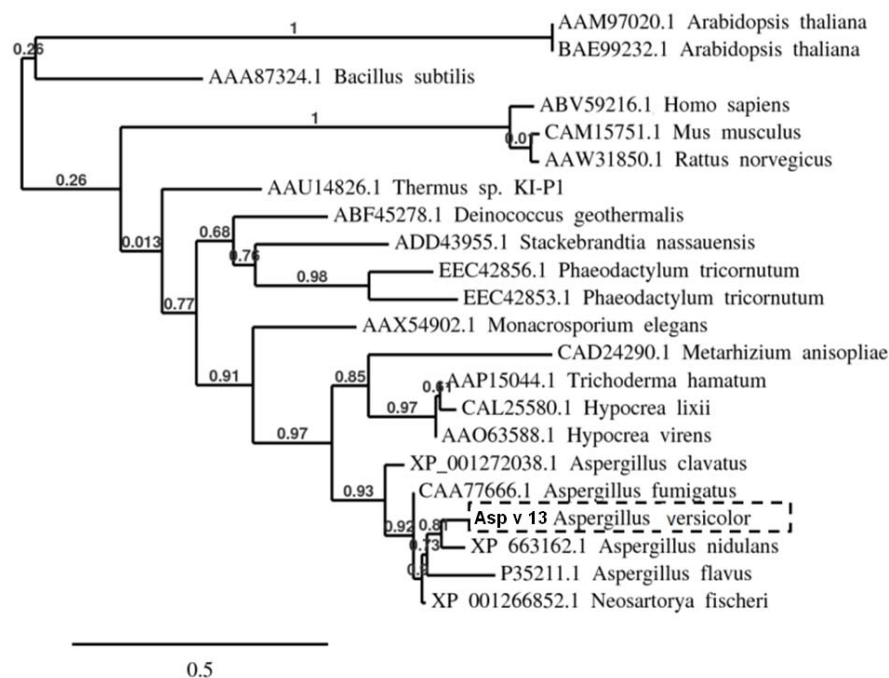


Fig. A.3. Phylogram type phylogenetic tree of Asp v 13 and other previously identified subtilisin-like serine protease families. Except Asp v 13 protein, all the other protein sequences were retrieved from GenBank. The phylogenetic positions of Asp v 13 is indicated by dashed square. The branch support values were indicated by their amino acid sequences.