

Original article

Molecular cloning and sequence analysis of full-length cDNAs encoding new group of Cyn d 1 isoallergens*

Background: Cyn d 1, the major allergen of Bermuda grass pollen, contains some acidic/basic isoforms. The *N*-terminal amino acid sequences of some acidic Cyn d 1 isoforms were found to be different from those of Cyn d 1 cDNA clones identified previously.

Methods: A predicted 17-meric oligonucleotide probe was designed to fish the unidentified isoallergen cDNAs out of BGP cDNA library. The reactive clones were isolated and verified by sequencing. Two of them were expressed in the yeast *Pichia pastoris* to obtain recombinant Cyn d 1 proteins.

Results: All four cDNA clones encode the full-length Cyn d 1 with mature proteins of 244 amino acid residues. A 97–99% identity was found among the deduced amino acids of these four clones while an 86% identity was elicited between the four clones and the ones previously identified. The predicted isoelectric focusing (pI) values of the newly identified Cyn d 1s are acidic while pIs of the previously identified Cyn d 1s are basic. The two recombinant acidic Cyn d 1 proteins possess the epitopes recognized by mouse and rabbit polyclonal anti-Cyn d 1 antibodies, and have human IgE-binding capacity as revealed by immunodot assay.

Conclusions: The present study identified full-length cDNAs encoding new isoallergens of Cyn d 1, and separated Cyn d 1 gene into an acidic group and a basic group.

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Bermuda grass (*Cynodon dactylon*) pollen (BGP) is an allergen of worldwide importance. The 30–34 kDa components extracted from BGP were identified as major allergens that reacted with more than 90% of human of IgE sera from patients allergic to BGP (1, 2). These components were classified as the group 1 allergen of BGP and were designated Cyn d 1 (2, 3). The isoforms of Cyn d 1 have been studied (4–7). A 2-D gel disclosed that Cyn d 1 contained several isoforms with pI values ranging from 5.6 to 7.3 or higher (5). In addition, some Cyn d 1 cDNA clones had been sequenced (6, 7). The sequences of cDNA clones were first identified by Smith et al. However, the cDNAs were truncated in the coding regions (6). Chang et al. then identified several full-length cDNAs of Cyn d 1 (7). These cDNAs encode identical Cyn d 1 *N*-terminal amino acid sequences of A/GDKPGPNITATYGSKWLE (7). On the other hand, we found that sequences of some acidic isoallergens showed substitutions of M, D, L, D for I, S, K, E (italicised in the aforementioned sequence), respectively

(5). This result suggests that another group of Cyn d 1 may exist. It has been documented that distinct isoforms of one allergen can display different immunological properties in terms of allergenicity (8). The present study designed and used an oligonucleotide probe to re-screen the BGP cDNA library in order to find this new group.

Material and methods

Construction of BGP cDNA library

The cDNA library of BGP was constructed previously (7). A poly(A)+RNA preparation of BGP was first reverse-transcribed using random and oligo (dT) primers. The cDNAs were cloned into the Lambda ZAP II expression vector by inserting the cDNAs into the *EcoRI* site. For convenience, the Lambda ZAP II vector was excised *in vivo* to generate a pBluescript SK-phagemid cDNA library by co-transfecting the Lambda ZAP II phage and a R408 helper phage (9) into *E. coli* XL 1-Blue host cells according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA).

Probe design

A 17-meric oligonucleotide probe (GCC ATG GGC GAC AAG CC) was designed based on the known *N*-terminal nucleotide sequence of Cyn d 1 cDNAs (5) except that the second codon ATC (for Leu) was substituted by ATG (for Met).

*The nucleotide sequence of cDNA clones 009, 008, 011 and 010 will appear in the GenBank Database under accession numbers. AF177379, AF159703, AF177378, and AF177380, respectively.

Isolation of cDNA clones for Cyn d 1

E. coli XL1-Blue was infected with the rescued phagemid. The infected bacteria were plated out on to Luria broth/ampicillin plates. Ampicillin-resistant colonies were transferred onto a nitrocellulose membrane and prepared for colony hybridization (10). The 17-meric oligonucleotide probe was ^{32}P -end-labeled with T4 polynucleotide kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as previously described (10). The membrane replicas were pre-hybridized at 65°C for 3 h in hybridization buffer [0.45 M sodium chloride, 0.045 M sodium citrate, 0.02 M potassium phosphate, pH 6.8, 0.1% (w/v) SDS, 0.02% (w/v) ficoll and 0.02% (w/v) polyvinylpyrrolidone] plus 100 µg/ml sheared and denatured herring-sperm DNA. The membrane replicas were then hybridized at 52°C for 8 h in 30 ml of hybridization buffer containing 5 pmole of the labeled oligonucleotide probe. Subsequent washes of the hybridized membrane filters were carried out in 6×SSC (1×SSC in 0.15 M NaCl/0.015 M sodium citrate) and 0.1% (w/v) SDS as follows: four times at 4°C for 5 min each, once at room temperature (5 min), and once at 52°C (2 min). The positive clones were detected by autoradiography, and were purified by isolating single-cell colonies.

Sequencing of cDNA clones

DNA sequencing was performed by the PCR/dideoxyl method using an ABI PRISM BigDye terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and an automatic sequencer (ABI PRISM 377–96 DNA Sequencer, Perkin-Elmer, Foster City, CA, USA). The two primers, T3 (TTAACCCTCACTAAAGGGA) and T7 (GGATATCACTCAGCATAAT), are located in the flanking regions of cDNA.

Expression of Cyn d 1 in the yeast *Pichia pastoris* system

This was performed with an EasySelect™ *Pichia* expression kit (Invitrogen Corp., San Diego, CA, USA) as previously described (7). Briefly, the cDNAs of mature Cyn d 1 proteins was amplified by PCR with created *EcoRI* site at the 5' end and *XbaI* site at the 3' end. The PCR products were purified, cut by restriction enzymes, and were inserted into the *EcoRI* and *XbaI* sites of pPICZa *Pichia* expression vector. The resulting Cyn d 1-pPICZa constructs were linearized with *PmeI*, and used to transform into *Pichia pastoris* strain KM71. The transformants were then grown for 2 days at 30°C in buffered minimal glycerol-complex medium. The expression of the recombinant Cyn d 1 proteins was induced with methanol (final volume 0.5%) for 3 days. The secreted recombinant proteins were isolated from media and analyzed by SDS-PAGE and immunodot assay as previously described (7, 11).

Dot-blot immunoassay

Dot-blots were prepared by applying 2 µg of the recombinant Cyn d 1 onto polyvinylidene difluoride membrane with a Bio-Dot apparatus (Bio-Rad, Richmond, CA, USA). After blocking, the blots were washed and then incubated with different antibodies including rabbit anti-Cyn d 1 antibodies (1:4000 diluted), mouse anti-Cyn d 1 antibodies (1:2500 diluted), anti-Cyn d 1 monoclonal antibodies (MAbs) 1–61 and 4–37, pooled normal human sera and sera from patients allergic to Cyn d 1 (1:4 diluted). After that, these blots were incubated with peroxidase conjugated second antibodies (i.e., goat antimouse, goat antirabbit or rabbit antihuman IgE antibodies in 1:2000 dilution) for 1 h. The blot was then washed thoroughly and incubated with ECL™ reagent (Amersham, Buckinghamshire, UK) for 1–2 min and exposed to X-ray films (Kodak, Rochester, NY, USA) for 5–20 s at room temperature with an intensifying screen.

Results

Isolation and verification of the new Cyn d 1 clones

The predicted 17-meric oligonucleotide probe was used to screen for the unidentified isoallergen cDNAs from the of BGP cDNA library. Several positive clones were isolated and sequenced. Some of the clones were found to be redundant. The nucleotide sequence and the deduced amino acid sequence of clone 009 are shown in Fig. 1. This clone is a Cyn d 1 clone as judged by the presence of the known *N*-terminal sequence in residues +1 to +20 of the deduced amino acid sequence (5). The cDNA encodes a protein of 244 amino acid residues. The sequence of residues –18 to –1, which leads to the *N*-terminal, is composed of hydrophobic amino acids and is a signal peptide. The motif Asn-X-Ser/Thr appearing at amino acid residues +9 to +11 is a putative glycosylation site.

The alignments of nucleotide sequences of three other positive clones, 008, 011, 010, with that of clone 009 indicates that good alignment and sequence conservation in the coding region among these clones except for some nucleotide polymorphisms (data not shown). Nucleotide sequences of the three other clones are available in GenBank database under accession numbers AF159703, AF177378 and AF 177380, respectively.

Analysis of the nucleotide and amino acid sequence of the two Cyn d 1 groups

The deduced amino acid sequences of these four clones (designated group A in Fig. 2) are aligned. In addition, four clones (designated group B in Fig. 2) of Cyn d 1 that we have isolated previously (7) are also arbitrarily selected and aligned in order to elucidate differences between these two groups.

Good alignment and sequence conservation in the coding region of the acidic group A are observed. There are only a total of 21 substitutions in the amino acid sequence for the proteins of clone 008, 011 and 010 (Fig. 2). An identity of 97–99% among the group A and 99% identity among the group B are found. However, the identity between members of these two groups dropped to 86%.

Moreover, the contents of acidic and basic amino acids of these two groups are different (Table 1). The net charge of charged amino acids of group A is –2. On the contrary, the net charge of B group is +6 to +8. Therefore, we hereby define group A and group B as acidic and basic group, respectively. These cDNA clones, together with the previously identified ones (6, 7), were reviewed by the Allergen Nomenclature Committee. The designated names of the related clones are also listed in Table 1.

The newly identified cDNAs encoding isoallergens of Cyn d 1

The coding region of two basic Cyn d 1 cDNA clones

GGCACGAGGGAAGAAGGAAGAAATGGCATCCTCCTCTCATGAACAGGCTATGCTCGGGC
 M L A A
 -18
 GGTGGCGGTGGTGGTCATCAATGGTTCGGCGGGCGCATGGTGTGCCATGGGCGACAAGCC
 V A V V L A S M V G G A W C A M G D K P
 -1 +1 +6
 AGGCCCAACATCACTGCAACCTACGGTGACAAGTGGCTGGATGCGAAGGCCACGTTCTA
 G P N I T A T Y G D K W L D A K A T F Y
 26
 CGGCAGCGACCCACGTGGCGCGCCCGGACGACCAAGCGCGCGGTGGCGATACAAGGA
 G S D P R G A A P D D H G G A C G Y K D
 46
 TGTGACAAAGGACCCCTTCGACGGCATGACGGATGCGGCAACGAGCCCATCTTCAAGGA
 V D K A P F D G M T G C G N E P I F K D
 66
 CGGTCTGGGCTGGGCTCCTGCTACGAGATCAAGTGAAGGAGCGCGGAGTGTCTCAGG
 G L G C G S C Y E I K C K E P A E C S G
 86
 TGAGCCCGTCCCTCATTAAGATCACTGACAAGAACTACGAGCACATGCGCGCTACCACTT
 E P V L I K I T D K N Y E H I A A Y H F
 106
 CGACCTTTCTGGCAAGGCGTTCGGTGCCATGCCAAGAAGGCGAGGAGGACAAGCTGGC
 D L S G K A F G A M A K K G E E D K L R
 126
 CAAGGCGCGGAGCTGTGCTGCGAGTTCGCGCGGTCAAGTGGAGTACCGGTCCGACAC
 K A G E L M L Q F R R V K C E Y P S D T
 146
 TAAGATCACTTCCACGTGGAAGAGGGCTCCAGCCCAATTACCTGGCGCTGCTGTGAA
 K I T F H V E K G S S P N Y L A L L V K
 166
 GTAGCCCGCGGAGGAGCAACATCGTGGTGTGACATCAAGCCCAAGGGCTCCGACGT
 Y A A G D G N I V G V D I K P K G S D V
 186
 GTTCTTCCCATGAAGCTGTTCATGGGGGCCATCTGGAGGATGAGCCCCCAAGCGCT
 F L P M K L S W G A I W R M D P P K P L
 206
 CAAGGGTCCCTTCAACATCCGCTCACCAGCGAGAGTGGCGCCATGTGGAACAGGAAGA
 K G P F T I R L T S E S G G H V E Q E D
 226
 TGTATATCCCGAAGACTGGAAGCCGACACCGTCTACAAGTCCAAGATCCAGTTCTGAGC
 V I P E D W K P D T V Y K S K I Q F *
 244

CTGATGTGCCCATAAGCAAGGTCCACACTAATAACCAACCTGATGACATCTTGTTCCTT
 TTTGCAAGAAACGGTTTATGGCATTCGATGCGTGACATACATATAATAACAAGCATGCAT
 GCGCGGTGAGGCTTTTCTCTCTTTCTTTCTACTATGTGTGACCTCCACAAATTCAT
 ATGAGAGTGTTTTGAGCAACAACATGTATATGTAATAAGTTCTCAGGATTGAGTGTGG
 CTAATATTTGTGAGCACTAGAGCAGAAAATATGTGAAAAACATCTCGTGCC

Figure 1. Nucleotide sequence of a new Cyn d 1 cDNA clone (009) and its predicted amino acid sequence. The amino acid residues are numbered on the right. The amino acid numbering starts at the N-terminal amino acid, alanine of Cyn d 1. The putative pre-peptide (signal peptide) consists of amino acid residues -18 to -1. The putative N-glycosylation site is underlined. Asterisk indicates the stop codon.

(4-8 and 9-4), and two acidic Cyn d 1 clones (009 and 011), were expressed in the yeast expression vector with a yield of approximately 10–20 mg/L. The presence of recombinant Cyn d 1 proteins in the culture medium was revealed by SDS-PAGE (Fig. 3A, lanes 2–5), together with native Cyn d 1 (Fig. 3A, lane 1). As shown, recombinant clones of 4-8, and 9-4 expressed secretory proteins with molecular weights of 27, 34, and 41–49 kDa (lanes 4 and 5). So did the recombinant clones of 009 and 011 (lanes 2 and 3). The SDS-PAGE profiles of the recombinant acidic Cyn d 1 proteins were similar to that of the basic Cyn d 1 proteins, but the

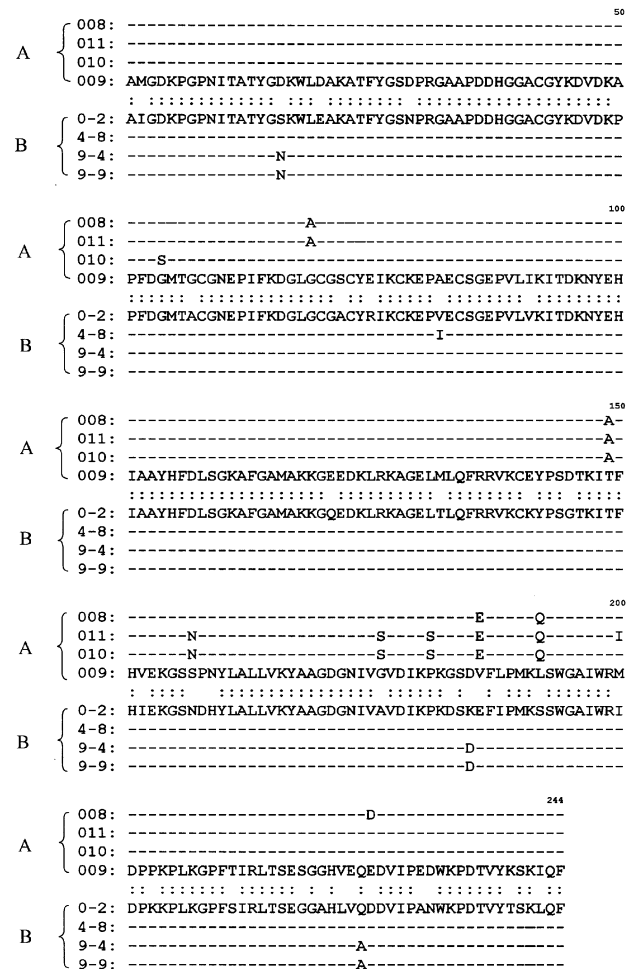


Figure 2. Amino acid sequence alignment of mature proteins for the two groups of Cyn d 1. The amino acid sequences of four newly isolated clones (group A) are aligned with those of four previously isolated clones of Cyn d 1 (group B) (7). Double dots indicate identical amino acids between clone 009 and 0-2. Dashed lines indicate the same amino acid as found in clone 009 (for group A) or 0-2 (for group B). The amino acid residues are numbered on the right. The putative N-glycosylation site is underlined.

molecular weights of the formers were slightly higher. The non-recombinant construct secreted no such proteins (lane 6).

The reactions between these recombinant Cyn d 1 proteins with polyclonal antisera and MAbs were

Table 1. The nomenclature and amounts of charged amino acids in the two groups of Cyn d 1

Clone	Nomenclature	Lys + Arg	Asp + Glu	Net charge
A	009 Cyn d 1.0204	33	35	-2
	008 Cyn d 1.0201	33	35	-2
	011 Cyn d 1.0202	33	35	-2
	010 Cyn d 1.0203	33	35	-2
B	0-2 Cyn d 1.0105	37	29	+8
	4-8 Cyn d 1.0106	37	29	+8
	9-4 Cyn d 1.0102	36	30	+6
	9-9 Cyn d 1.0103	36	30	+6

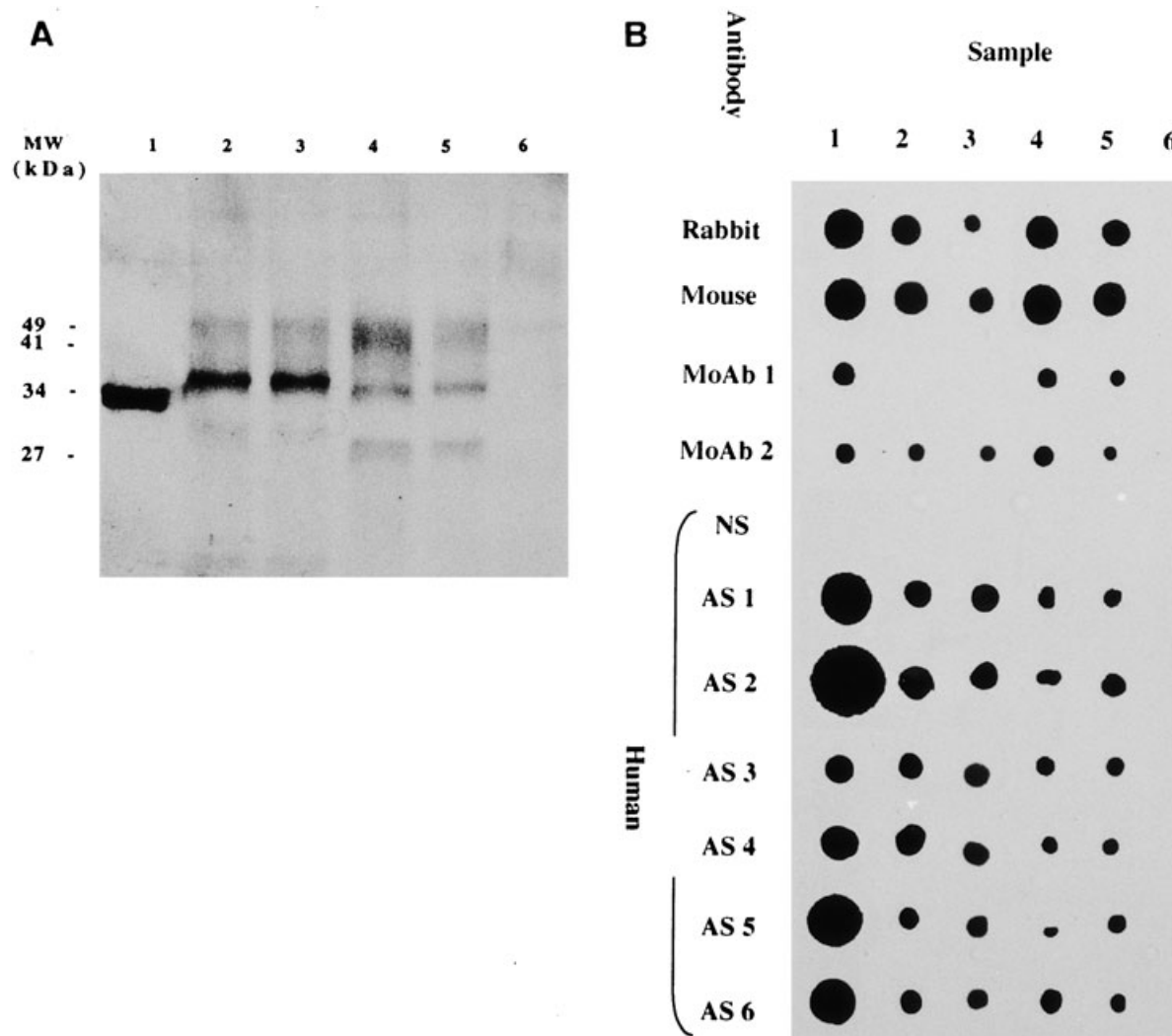


Figure 3. SDS-PAGE and immunodot profiles of recombinant Cyn d 1 protein. Purified natural Cyn d 1 (lane 1), recombinant proteins of acidic Cyn d 1 009 (lane 2) and 011 (lane 3), recombinant proteins of basic Cyn d 1 4–8 (lane 4) and 9–4 (lane 5) and secretory proteins from non-recombinant yeast control (lane 6) were separated by SDS-PAGE (*A*), or transferred onto polyvinylidene difluoride membranes (*B*) to perform an immunodot with rabbit anti-Cyn d 1 polyclonal Ab, mouse anti-Cyn d 1 polyclonal Ab, anti-Cyn d 1 MAb 1 (1–61), anti-Cyn d 1 MAb 2 (4–37), IgE in the pooled normal human sera (NS), and IgE in the serum of BGP-allergic patients (AS1~AS6).

delineated with an immunodot assay (Fig. 3B). As shown, polyclonal anti-Cyn d 1 rabbit and mouse antibodies reacted with all these four recombinant proteins. Interestingly, MAb 1 (1–61), reacted only with the basic recombinant protein while MAb 2 (4–37) reacted with all the recombinant Cyn d 1 proteins. In addition, all these four recombinant proteins (009, 011, 4–8, 9–4) reacted with IgEs of sera of patients allergic to BGP (i.e., AS1~AS6) but not with IgEs of pooled normal sera (NS).

Discussion

Using 2-D gel analysis, Cyn d 1 had been demonstrated to contain some acidic and basic isoallergens; the acidic Cyn d 1 s were with a slightly higher molecular weight than that of the basic Cyn d 1 (5). However, the 10

previously cloned full-length cDNAs belong to the basic isoallergens (7) (group B by our definition). The present study identified new acidic Cyn d 1 (group A) and demonstrated it to be isoallergens as the recombinant proteins derived from the cDNAs of 009 and 011 did react with anti-Cyn d 1 antibodies and sera from patients allergic to Cyn d 1 (Fig. 3B). The finding that molecular weights of the acidic recombinant Cyn d 1 proteins were slightly higher than those of the basic recombinant Cyn d 1 proteins (Fig. 3A) correlated well with the data obtained from the protein level (5). The identification of this acidic Cyn d 1 should be of academic importance since the group 1 allergens Lol p 1 of rye grass pollens are solely acidic proteins (12, 13). An identity of 69% is found between the acidic Cyn d 1 and Lol p 1.

Besides the difference in net charge for group A and B

(see Table 1), other evidences also indicated that they are separate groups. It was found that 97–99% of identity among group A and 99% of identity among group B. However, identity between members of these two groups drops to 86%. Moreover, the signal peptide of group A and B Cyn d 1 are composed of 18 (see Fig. 1) and 26 amino acid residues (7), respectively. The group A Cyn d 1 mature proteins are composed of 244 amino acid residues. Some group B Cyn d 1 s have two additional GA residues at the C-terminus (7); this has not been found in the acidic Cyn d 1. Taken together, the present study demonstrated the sequence polymorphisms of Cyn d 1 are more complex than previously had expected (5). However, amino acid variations in Cyn d 1 are fewer as compared to Pha a 5 of which variations are in the range in 19% to 60% (14).

In addition to the four group A Cyn d 1 cDNA clones, other four cDNA clones had been sequenced (data not shown). All of their mature proteins are with the N-terminal amino acid sequences AMGDKP and are predicted to be with acidic pI value. In comparison with the 10 previously identified basic Cyn d 1 cDNA clones, it appears that Cyn d 1 isoallergens with the N-terminal sequence of AIGDKP are the basic group and Cyn d 1 proteins with N-terminal sequence of AMGDKP are more likely to be the acidic group. Although Cyn d 1 cDNA clones with such a N-terminal amino acid sequence had not been identified by Smith et al. (6), the deduced amino acid sequence of their Cyn d 1.3 which derived from two partial Cyn d 1 cDNA clones were 98.9% identical to that of the clone 010 in the present study (Fig. 2).

The biological functions of BGP Cyn d 1 are still unknown. According to sequence analysis of the Cyn d 1 cDNAs identified, they all contain a signal peptide and a conserved N-glycosylation site at amino acid residues of +9 to +11 (Fig. 2). No transmembrane domain has been found. It appears that they are secretory glycoproteins that meet the water-extractable nature of Cyn d 1. There are a total of 21 substitutions in amino acids (see Fig. 2) and five-fold (i.e., 104) more

nucleotides substitutions in the coding regions of clones 008, 011 and 010. It implies that most missense mutations are unfavourable for propagation and/or survival of BGP.

Our BGP cDNA library was generated from pooled BGP collected in an open field (7). Whether or not those isoallergens are derived from closely related gene families and/or just are allelic polymorphisms of the same gene (or genes) in the BGP population, allergists have to face a diversified allergenicity attributed to the amino acid variations in these two groups. It is well known that amino acid substitutions of a protein may change its antigenicity. Although the amino acid variations between these two groups of Cyn d 1 are only 14%, it caused the change of their charges (Table 1) and will most likely lead to the difference of their structures which would then result in the loss or attenuation of some epitopes. This is proven by the finding that MAb 1–61 is capable of reacting with basic Cyn d 1 recombinant proteins 4–8 and 9–4 but not with acidic Cyn d 1 recombinant proteins 009 and 011 (Fig. 3B).

The immunological properties of isoforms of some allergens have been well characterized (8, 15–17). Some isoforms of Bet v 1 had been disclosed to be with less IgE-binding capacity and stronger T cell stimulating effect (15), and these hypoallergenic isoforms were suggested to be better than those of others as targets for specific immunotherapy (18–20). The immunological properties of Cyn d 1 isoforms should be furthermore disclosed in the future; and the acidic and basic recombinant Cyn d 1 proteins obtained in the present study can serve as useful reagents to perform the study.

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