

Molecular cloning of cDNA coding for the 68 kDa allergen of *Penicillium notatum* using MoAbs

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Summary

To characterize the 68 kDa allergen of *Penicillium notatum* (also known as *P. chrysogenum*), a molecular antibody (MoAb) (P40) was previously generated. For cDNA cloning, three more MoAbs (3F, 5A3, 5G2) were generated in the present study. A mixture of all the four MoAbs was used in cloning of the gene coding for the 68 kDa allergen from a λ gt11 cDNA library of *P. chrysogenum*. A cDNA clone (A6) with DNA insert of about 0.5 kb which encodes for the 3'-terminal nucleotide sequence of the 68 kDa allergen was obtained. The cloned sequence contained two putative N-glycosylation sites. The reduction in molecular weight from 68 to 62 kDa in immunoblotting after treatment of the crude extract of *P. notatum* with N-glycosidase F indicates that the 68 kDa allergen is a glycoprotein. Nucleotide sequence determination showed that 188 (54%) of the 348 nucleotides of the cDNA sequence obtained were identical to the same region of the nucleotide sequence of the beta-N-acetylglucosaminidase gene of *Candida albicans*. Although the cDNA clone obtained did not encode the full-length gene of the 68 kDa allergen, polypeptide expressed from the A6 cDNA showed positive immunological reactivities to all four MoAbs used in the cloning experiment and to IgE antibodies in sera of asthmatic patients. There was a loss of immunoblotting activity to the 68 kDa component after absorption of MoAb P40-containing culture supernatant with filters blotted on plaque lawns of cDNA clone A6. Moreover, the immunoblotting activity remained when the MoAbs affinity-purified with filters containing polypeptides encoded by the cDNA insert of clone A6 were used. These two observations indicate that clone A6, which encodes 117 amino acid residues of a putative 560-residue polypeptide, is a cDNA clone of the 68 kDa component of *P. notatum*. In conclusion, results obtained from cloning and characterization of a partial cDNA clone described in this study suggest that the 68 kDa allergen of *P. notatum* is a beta-N-acetylglucosaminidase.

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Introduction

Rapid advances have been made in the past few years on allergen characterization through molecular biological approaches. For example, at least seven different groups of mite allergens have been characterized based on known cDNAs, N-terminal sequences or enzymatic

functions [1]. The nucleotide or amino acid sequences for allergens, such as pollen allergens of Amb a 1, Lol p 1 (group 1) and Bet v 1 [2–4], animal allergen of Fel d 1 [5], insect allergens of Api m 1 and Dol a 5 [6,7] and food allergens of Gad c 1 and Pen a 2 [8,9], have also been determined. Data obtained provide valuable information concerning major features of these important allergens. However, among numbers of fungal allergens, only the sequence data of Asp f 1, the 65 kDa allergen of *A. fumigatus*, the 40 kDa allergen of *C. albicans* and Alt a 1

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[10–13] are available. Since *Penicillium* has been shown to be one of the fungal genera frequently found to be associated with allergy [14,15], we have identified previously by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that the 68 kDa component is an important allergen of *P. notatum* [16]. A MoAb (P40) against this allergenic component has been generated [17]. In the present study, we report the preparation of additional MoAbs and the cloning and sequence analysis of the gene encoding for the 68 kDa allergen of *P. notatum*. Results obtained suggest that this allergen is a beta-*N*-acetylglucosaminidase.

Materials and methods

Allergenic extracts

The ground and freeze-dried fungal preparations were obtained from Allergon AB (Engelholm, Sweden). They were four species of *Penicillium* and four species of *Aspergillus*. The *Penicillium* species used were *P. notatum*, *P. frequentans*, *P. roseopurpureum* and *P. roquefortii*. The *Aspergillus* species used were *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. Fungal mats of *P. citrinum* (isolated from the air of Taipei area, [18]) containing both spores and mycelia which were collected after its growth in Czapek yeast extract [19] for 3 weeks at room temperature were also used in the present study. Crude extracts of different fungi were prepared by extracting 5 g of each species with 100 ml of phosphate-buffered saline (containing 85 mmol/l of NaCl, 2.6 mmol/l of KH_2PO_4 , and 49 mmol/l of Na_2HPO_4 , pH 8.0) for 36 h at 4°C with constant shaking. After centrifugation, the supernatant was dialysed against phosphate buffer (10 mmol/l, pH 7.2) for 48 h at 4°C. After sterile filtration (0.2 µm; Sartorius, Hayward, CA, USA), the extract was lyophilized in aliquots and stored at 4°C. The protein content of the extract was determined by the dye-binding method of Bio-Rad (Richmond, CA, USA).

Development of MoAbs

MoAbs against the 68 kDa component of *P. notatum* were prepared essentially as described previously [17]. The isotype of the MoAbs was determined by ELISA with class-specific antisera (Southern Biotechnology Associates, Inc., Birmingham, AL, USA). Reactivity of MoAbs to different fungal species was analysed by SDS-PAGE-immunoblotting as described previously [17].

Periodate oxidation

Periodate oxidation of antigens was performed as described by Woodward *et al.* [20]. The antigens of *P.*

notatum coated onto a microtitre plate were exposed to various concentrations of sodium m-periodate (May & Baker, Dagenham, UK). The activity of MoAbs to antigens treated by periodate oxidation was then determined by ELISA as described before [17].

Cloning and molecular techniques

A λgt11-based cDNA library of *P. chrysogenum* (*P. notatum*, cat. no. YL1003b) was purchased from the Clontech Laboratories, Inc. (CA, USA). The library was constructed from polyA⁺ RNA prepared from culture mats of *P. chrysogenum*. The library contained 1.4×10^6 independent clones with the size of the cDNA in the range of 0.6–4.0 kilobase pairs (kb) with an average size of 1.2 kb. λgt11 clones containing DNA sequences of the 68 kDa antigen were obtained by immunoscreening of the IPTG-induced library using mixtures of MoAbs generated as described previously [12]. The cDNA insert in the clone obtained (A6) was subcloned into the EcoRI site of the plasmid vector pGEM-3Zf(+). The nucleotide sequences were determined by the dideoxy sequencing procedure as described previously [12].

Deglycosylation

Deglycosylation of *Penicillium* components was performed according to the protocol recommended by the manufacturer (Boehringer Mannheim, Mannheim, Germany). The sample (30 µg of the crude extract of *P. notatum*) was boiled in 10 µl of 1% SDS for 2 min. After the addition of 40 µl of sodium phosphate buffer (20 mmol/l, pH 7.2, containing 10 mmol/l of sodium azide, 50 mmol/l of EDTA and 0.5% v/v of Nonidet P-40), the sample was boiled again for 2 min. After cooling, 1.2 units of *N*-glycosidase F (Boehringer Mannheim) were added into the tube. The sample was then incubated for 16 h at 37°C. For a control, the same amount of sample was treated and incubated as described above except that no enzyme was added. The enzyme-treated and non-treated samples were analysed by SDS-PAGE and immunoblotting with MoAb P40.

Immunological characterization of cDNA clones obtained

To characterize the cDNA clone obtained, IgE plaque immunoassay was conducted by the standard procedure [21] using 1500 p.f.u. on 15 cm petri dishes and sera from allergic patients diluted 1/2 in *E. coli* lysate. IgE reactivity was detected with alkaline phosphatase-labelled monoclonal anti-human IgE (Pharmingen, San Diego, CA, USA) and developed using the alkaline

phosphatase conjugate substrate kit (Bio-Rad, catalog number 170-6432). To identify the corresponding native component, immunoblotting of the crude extract of *P. notatum* was performed using MoAb P40-containing supernatant which has been incubated overnight with a nitrocellulose filter blotted on a plaque lawn of the cDNA clone obtained (A6) as the absorbant. Furthermore, MoAbs affinity-purified by elution of the Abs adsorbed onto the same absorbant filter described above, with a solution of 0.1 M glycine, 0.15 M NaCl, pH 2.6 [21], were also used in immunoblotting to identify the corresponding natural allergen. For control, nitrocellulose filters blotted on plaque lawns of a λ clone randomly picked from the *P. chrysogenum* cDNA library were used as control absorbants.

Results

Development of MoAbs

To clone the 68 kDa allergen of *P. notatum*, more MoAbs were generated in the present study. Cell fusion between spleen cells of BALB/c mice immunized with the crude extract of *P. notatum* and NS-1 cells resulted in a series of hybridomas secreting MoAbs against antigens of *P. notatum*. After screening by immunoblotting and cloning by limiting dilution, three MoAbs (3F, 5A3, 5G2) reacting with the 68 kDa component of *P. notatum* were obtained (Fig. 1). All MoAbs showed isotypes of mouse IgG1 with k light chain.

The reactivity of MoAbs to different *Penicillium* and *Aspergillus* species was analysed by immunoblotting. All three MoAbs generated in the present study showed a similar reaction pattern to different fungal species tested which is different from the one given by MoAb P40 (Table 1). The crossreacting antigens in different fungal species recognized by MoAbs had a similar mol. wt. of about 68 kDa (data not shown).

To determine if the epitopes recognized by these MoAbs were associated with carbohydrates, the binding activity of MoAbs to periodate-treated antigens was examined by ELISA. Epitopes on the 68 kDa molecule recognized by MoAbs 3F, 5A3 and 5G2 were relatively resistant to treatment of periodate oxidation with concentrations of periodate of up to 20 mM (data not shown). Since the epitope recognized by MoAb P40 was also periodate-resistant [17], all four MoAbs were used in the cDNA cloning of the 68 kDa allergen.

cDNA cloning, sequencing and immunological analysis

Using a mixture of the four MoAbs (P40, 3F, 5A3, 5G2) as an immunological screening probe, 1×10^6

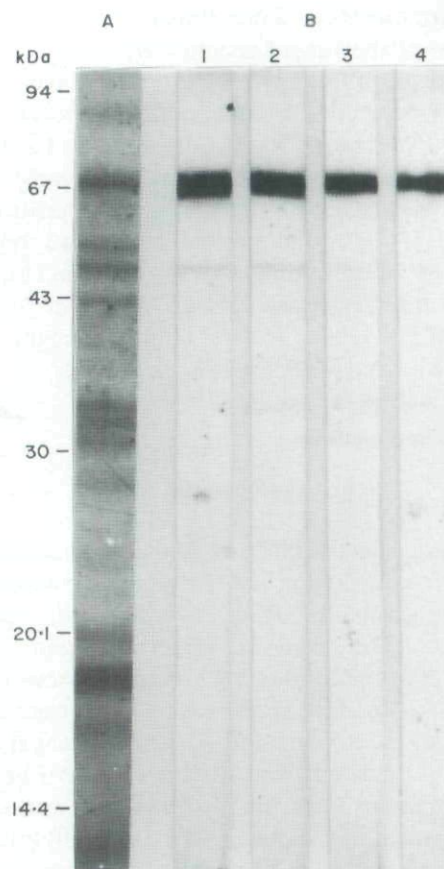


Fig. 1. Immunoblotting reactivity of MoAbs to the crude extract of *P. notatum*. Panel A shows the Coomassie blue-stained membrane blotted with components of *P. notatum* separated by SDS-PAGE. Strips 1–4 of panel B show immunoblotting activity of MoAbs P40, 3F, 5A3 and 5G2, respectively to components of *P. notatum*.

Table 1. Reactivities of MoAbs to different species of *Penicillium* and *Aspergillus* analyzed by SDS-PAGE-immunoblotting

Mould species	Reactivities of MoAbs			
	P40	3F	5A3	5G2
<i>Penicillium</i> species				
<i>P. notatum</i>	+	+	+	+
<i>P. citrinum</i>	–	+	+	+
<i>P. frequentans</i>	+	+	+	+
<i>P. roseopurpureum</i>	+	+	+	+
<i>P. roquefortii</i>	–	+	+	+
<i>Aspergillus</i> species				
<i>A. fumigatus</i>	+	+	+	+
<i>A. flavus</i>	+	+	+	+
<i>A. niger</i>	+	–	–	–
<i>A. terreus</i>	+	+	+	+

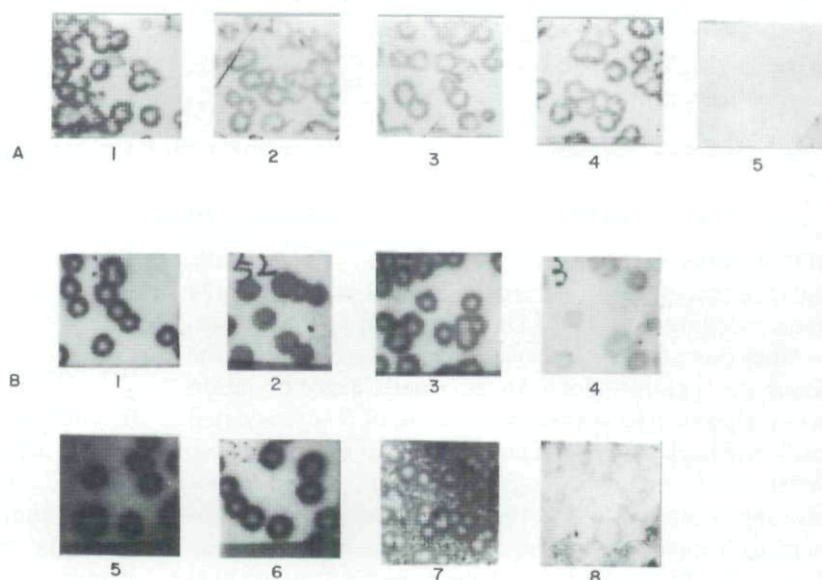
plaque-forming units of an IPTG-induced cDNA library of *P. chrysogenum* were screened. After three rounds of plaque purification, a single and consistently positive plaque (A6) was obtained. The antigenicity and the IgE-binding reactivity of the polypeptide encoded by cDNA clone A6 were examined by plaque immunoassay using individual MoAb and sera from asthmatic patients. Four MoAbs reacted individually with the lysate of cDNA clone A6 (Fig. 2, part A, Nos. 1–4). For negative control, a MoAb against the 40-kDa allergen of *C. albicans* was used (Fig. 2, A-5). Lysates of clone A6 also reacted with IgE antibody in sera of seven different asthmatic patients (Fig. 2, part B, Nos. 1–7) which had previously been shown by immunoblotting to contain IgE antibody to the component of *P. notatum* with a mol. wt. of about 68 kDa (data not shown). For control, enzyme colour reaction was not detected when the lysate of clone A6 was reacted with serum from a mite-allergic patient (Fig. 2, B-8).

An insert of about 0.5 kb of clone A6 was subcloned into plasmid pGEM-3Zf(+) at the EcoRI cloning site. The nucleotide sequence of subclones was determined by direct dideoxy sequencing of the plasmid DNA. The nucleotide sequence and the translated amino acid sequence of the insert DNA (474 bp) of cDNA clone A6 are shown in Fig. 3. The insert DNA is truncated at the 5' end and contained two cysteines and two putative *N*-glycosylation sites (Fig. 3). The 'AATAAA' typical polyadenylation signal sequence for mammalian genes is not found within 10–30 bases upstream from the polyA tail in clone A6. Instead, AT-rich sequences are observed as in other yeast genes (Fig. 3). When this nucleotide

sequence was compared with the DNA database of the IntelliGenetics, Release 5.4 (IntelliGenetics, Inc., Mountain View, California, USA), 188 of the 348 nucleotides (54%) of the sequence of the cDNA insert were found to be identical to the same region of the nucleotide sequence of the beta-*N*-acetylglucosaminidase (HEX1) gene of *Candida albicans* (accession no. L26488). When the amino acid sequences encoded by these two nucleotide sequences were compared (amino acid residues 447–562 of the HEX1), an amino acid sequence identity of 61% (71/116) was observed (Fig. 3). Many of the nucleotide differences in the two sequences occurred at the third position of the codon, often resulting in no amino acid change.

To identify the corresponding native component, immunoblotting of the crude extract of *P. notatum* was performed using P40-containing supernatant which had been absorbed with the lysate of clone A6 and MoAbs affinity-purified by using the absorbant filter blotted on a plaque lawn of clone A6. Loss of immunoblotting activity to the 68 kDa component of *P. notatum* was observed when the absorbed P40-containing supernatant was used (data not shown). For control, the immunoblotting activity was still observed after absorption of the P40-containing supernatant with a lysate of a cDNA clone randomly picked from the cDNA library of *P. chrysogenum* (data not shown). Furthermore, the immunoblotting activity to the 68 kDa component was still observed when the affinity-purified MoAbs were used (data not shown). However, enzyme colour reactivity was not detected when MoAbs affinity-purified with filters blotted on plaque lawns of a randomly picked

Fig. 2. Plaque immunoassay of reactivities of MoAbs and IgE antibodies in sera of asthmatic patients to IPTG-induced plaques of cDNA clone A6. Segment nos. 1–4 of part A show results using MoAbs P40, 3F, 5A3 and 5G2, respectively. For control, one MoAb against the 40 kDa allergen of *C. albicans* was used (A-5). Segment numbers 1–7 of part B show results when sera from seven different asthmatic patients with IgE Abs to components of *P. notatum* with a mol. wt. of about 68 kDa were used. For control, serum from a mite-allergic patient was used (B-8).



A6:	GGG CCC TAC AAG ACC TGG CAG CGC ATC TAC GAC TAC GAC TTC CTC	45
	Gly Pro Tyr Lys Thr Trp Gln Arg Ile Tyr Asp Tyr Asp Phe Leu	
HEX1:	..A ..T ..T ... T.T .AT ..A ..A ..T ... A.. .TT ..T ... ACT	
	Ser Tyr Asn Phe Thr	
A6:	ACG AAT CTC ACT TCC TCC GAA GCG AAC GAC ATT ATC GGC GCC GAG	90
	Thr Asn Leu Thr Ser Ser Glu Ala Asn Asp Ile Ile Gly Ala Glu	
HEX1:	G.T ... T.G ..A GAA A.A ... AAA ... C.T G.. T.A ..T CGT ..A	
	Ala Glu Thr Lys His Val Leu Arg	
A6:	GCT CCT TTG TGG TCG GAG CAG GTC GAC GAT GTG ACC GTC TCC AGC	135
	Ala Pro Leu Trp Ser Glu Gln Val Asp Asp Val Thr Val Ser Ser	
HEX1:	... G.. ..A A ..T ..T TC. ACT GT. T.G A.T .CA	
	Ala Ser Thr Val Leu Thr Thr	
A6:	GTG TTC TGG CCT CGC GCT GCT GCT CTG GGT GAG CTT GTC TGG TCT	180
	Val Phe Trp Pro Arg Ala Ala Ala Leu Gly Glu Leu Val Trp Ser	
HEX1:	AA. A.A A ..T A.. ..A ..A ..T .C. ..A T.G ACT ... AGC	
	Lys Ile Thr Ala Thr	
A6:	GGT AAC CGT GAC GCT GCG GGT AGA AAG CGT ACC ACC AGC TTT ACT	225
	Gly Asn Arg Asp Ala Ala Gly Arg Lys Arg Thr Thr Ser Phe Thr	
HEX1:	..G ... AAG ..T T.C AAC ..A CAC C.C A.A GGG TAT GAA ..C ...	
	Lys Ser Asn His His Gly Tyr Glu	
A6:	CAG CGT ATT CTG AAC TTC CGT GAA TAC CTC GTT GCC AAT GGT GTG	270
	Gln Arg Ile Leu Asn Phe Arg Glu Tyr leu Val Ala Asn Gly Val	
HEX1:	..A A.A ..A T.A A.G ..G ..T T.A ..C AAA TTA ..A TAC	
	Lys Leu Tyr	
A6:	ATG GCT GCT GCT CTT GTG CCG AAG TAT TGT CTG CAG CAC CCT CAT	315
	Met Ala Ala Ala Leu Val Pro Lys Tyr Cys Leu Gln His Pro His	
HEX1:	GG. .T. T.. C.. T.G A ..A ..C ... T.. .TC A.T ..A ..C	
	Gly Val Ser Pro Leu Asn	
A6:	GCT TGC GAC CTC TAT AAA AAC CAG ACT GTA ATG TCT <u>TGATGTGGTT</u>	362
	Ala Cys Asp Leu Tyr Lys <u>Asn Gln Thr</u> Val Met Ser	
HEX1:	T ..T T.G ..C T .CA C.A ..T TAT <u>.GA</u>	
	Pro Pro Tyr	
A6:	AAGCTGGACTGCTAGTGAGCCTTACAACGCTGTTCTGTCGTATATACTTATTCTATC	421
A6:	TTCGATACCCAATTCCATTGGAATTTCTCCAGGAAAAAAAAAAAAAAAAAAAAA	474

Fig. 3. Nucleotide and amino acid sequences of the insert cDNA of clone A6 (top two lines) and the beta-N-acetylglucosaminidase (HEX1) gene of *Candida albicans* (bottom 2 lines). For HEX1, only the nucleotide and the amino acid residues that are different from those of A6 are listed. The HEX1 amino acid sequence shown is from residues 447–562. Underlined sequences are N-glycosylation sites and the TGA stop codon.

cDNA clone were used (data not shown). These results indicate that clone A6 did contain partial sequence of the gene encoding for the 68 kDa component of *P. notatum*.

Since potential N-glycosylation sites were found in the sequence of cDNA clone A6, enzymatic deglycosylation was undertaken to test for the presence of N-glycosylated carbohydrates on the 68 kDa component of *P. notatum*. After treatment with N-glycosidase F, an immunoblotting reactivity of MoAb P40 to *Penicillium* component with mol. wt. of about 62 kDa was observed (Fig. 4 lane 2). The component with a mol. wt. of 68 kDa

was still shown to be reactive to MoAb P40 in control experiment containing buffer only (Fig. 4 lane 1).

Discussion

In addition to MoAb P40, three more MoAbs against the 68 kDa allergen of *P. notatum* were generated in the present study. Results from immunoblotting demonstrated that all three MoAbs showed a similar cross-reactivity pattern to nine different *Penicillium* and *Aspergillus* species, most of which have been reported

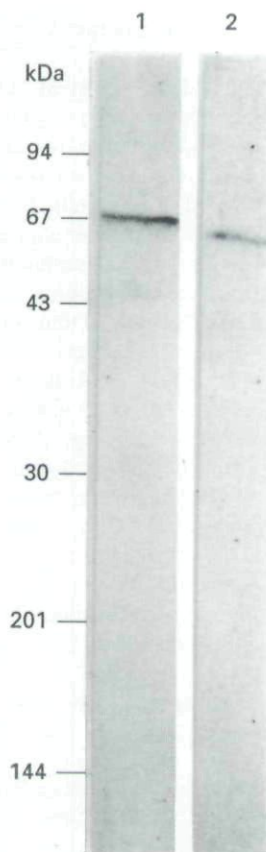


Fig. 4. Immunoblotting reactivity of MoAb P40 to components of *P. notatum* treated with buffer containing *N*-glycosidase F (strip no. 2) or with buffer only (strip no. 1).

as causative agents of human allergy [22]. The pattern is different from the one when MoAb P40 was used. Thus, at least two different epitopes on the 68 kDa component were probably recognized by these MoAbs. The reason for the inclusion of *P. citrinum* in the crossreactivity study was that it has been identified to be the most prevalent air-borne *Penicillium* species in Taipei area [18]. Three MoAbs showed positive while MoAb P40 showed negative reactivity in immunoblotting to components of *P. citrinum*.

A cDNA clone (A6) from a λ gt11 cDNA library of *P. chrysogenum* was obtained using a mixture of the four MoAbs generated as an immunological probe. Molecular analysis revealed sequence identities of 54% and 61%, respectively in the nucleotide and the amino acid sequences of the insert DNA of clone A6 to the same region of the beta-*N*-acetylglucosaminidase (HEX1) gene of *Candida albicans*. The *N*-acetylglucosaminidase catalyses the hydrolysis of *p*-nitrophenyl-beta-*N*-acetylglucosamine, *N,N'*-diacetylchitobiose and *N,N',N''*-triacetylchitotriose [23]. It is rapidly secreted

to the periplasm when the enzyme is induced either during yeast growth at 28°C or germ-tube formation at 37°C [23]. Results obtained from SDS-PAGE and gel permeation chromatography suggest that the native HEX1 of *Candida albicans* is a monomer with a mol. wt. of 66 000 Da [23] which is close to the mol. wt. of 68 kDa for the allergen of *P. notatum* under investigation.

Although the insert cDNA of clone A6 is truncated at 5' end, data obtained from immunoblotting using MoAb-containing supernatant absorbed with the lysate of clone A6, as well as Abs affinity-purified using filters blotted with polypeptide encoded by clone A6, indicate that A6 is a cDNA clone of the 68 kDa component of *P. notatum*.

There are two putative *N*-glycosylation sites in the amino acid sequence of clone A6. However, only one glycosylation site is found in the corresponding sequence of HEX1 (Fig. 3). The enzyme *N*-glycosidase F cleaves *N*-linked oligosaccharides between *N*-acetylglucosamine and the asparagine residue. The reduction in mol. wt. after treatment with *N*-glycosidase F indicates that the 68 kDa component of *P. notatum* is a glycoprotein. Since other putative *N*-glycosylation sites can still be found in the amino acid sequence of HEX1, at present, it is uncertain which is (are) the functional glycosylation site(s) for the 68 kDa component of *P. notatum*.

Although clone A6 did not contain the full-length genome of the 68 kDa component of *P. notatum*, the polypeptide encoded by the cDNA insert showed positive reactivity to individual MoAb used in molecular cloning and to IgE antibodies in sera from asthmatic patients. Thus, antigenic/allergenic epitopes were included in the polypeptide encoded by A6. However, all four MoAbs used in the present study showed negative immunoblotting reactivity to components of *Candida albicans* (data not shown). Thus, although an amino acid sequence identity of 61% was found in corresponding sequences of *P. notatum* and *Candida albicans*, there are still epitopic domains unique for each fungus.

In conclusion, results obtained from this study suggest that the 68 kDa allergen of *P. notatum* is a beta-*N*-acetylglucosaminidase. The cloning, sequencing and expression of the full-length gene of this enzyme are being undertaken to obtain more information concerning this *Penicillium* allergen.

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