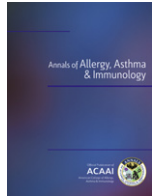




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Letter

Glucoamylase is a potential allergen in products expressed in *Neurospora crassa*

Neurospora crassa, a well-characterized filamentous fungus, has been proposed as a vaccine production platform because of low cost, rapid expression, and molecular flexibility. Vaccine antigens are expressed and secreted by *N. crassa* in liquid culture, and the supernatant is then filtered and processed.¹ Because such vaccines are intended for human use, the presence of fungal allergens that might co-purify with vaccine antigens needs to be ascertained and minimized. Our goal was to screen *N. crassa* conditioned medium (CM) for the presence of such potential allergens. In the process we identified the presence of *Neurospora* glucoamylase I precursor, a putative allergen of possible clinical significance.

Three-day *N. crassa* CM and growth media alone (containing Soytone, maltose, glucose, 2-(N-morpholino) ethanesulfonic acid buffer, Tween 80, and L-arginine) were used as antigen source and control. A highly complex (1.42×10^9) naïve human single-chain variable fragment (scFv) library was panned and screened against CM. Eighty specific clones were obtained after 3 rounds of bio-panning and enrichment. Sequence analysis revealed 4 unique clones (NC3, NC14, NC65, and NC81) with greatest divergences in CDR2 of V_H and CDR3 of V_L chains (eFig 1A). Selected scFv antibodies were expressed and purified from *Escherichia coli* with hexahistidine at the N-termini and c-myc at the C-termini by nickel affinity chromatography (eFig 1B). All 4 scFv antibodies were reactive with a ~70 kDa band (eFig 1C).

To identify the approximately 70-kDa band, immunoprecipitation was performed. After desalting, NC65 was bound to protein-L resin for 4 hours followed by incubation with CM overnight at 4°C. The resin was washed and bound proteins were eluted with sodium dodecyl sulfate sample buffer. Eluted fractions were analyzed using Coomassie stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Fig 1A). The target protein (indicated by arrow in Fig 1A) was excised and subjected to matrix-assisted laser desorption/ionization (MALDI) analysis. The obtained peptide sequences matched with the glucoamylase I precursor protein of *N. crassa* (XP_956966.1) (eTable 1 and eFig 2A).

Amylases and glucoamylases used in industrial settings^{2,3} are known to cause allergy and asthma, and other structurally similar glucoamylase allergens (Asp n 14 and Sac c 1.0101) have already been identified. To determine the antigen specificity of NC65, several mold extracts and enzymes were evaluated by immunoblot using NC65 as the primary antibody (eFig 2B). Protein bands that were recognized included α -amylase of *Aspergillus oryzae* (lane 1) and several bands in a baker's yeast (*Saccharomyces cerevisiae*) extract, most notably a band at 60 kDa (lane 8).

To assess whether glucoamylase I precursor and the other fungal proteins in *N. crassa* CM are potential allergens, we examined immunoglobulin E (IgE) binding to *N. crassa* CM by IgE immunoblot, using human sera from 2 sources: IgE anti-amylase positive sera collected from bakery workers with occupational asthma and allergy symptoms,⁴ and samples from individuals with IgE against *A. fumigatus* (obtained from PlasmaLab International, Everett, Washington). IgE in each of the sera tested recognized 70-kDa and 65-kDa proteins (Fig 1B and C), as well as, for a few of the sera, additional low-molecular-weight bands. Peptide sequence analysis of 2 major bands and 2 low-molecular-weight protein bands (~17 kDa and ~24 kDa) obtained from Coomassie-stained gel indicated them to be glucoamylase and its degradation products (data not shown).

Fungi produce and release into the environment a variety of allergens in varying amounts. Fungal enzymes used in industrial settings may elicit occupational allergic diseases,² among them bakers' asthma. α -Amylase derived from *A. oryzae* (Asp o 2) is frequently used to improve the quality of bread-making dough, and is known to induce IgE sensitization in exposed workers.^{2,5,6}

Like α -amylase, glucoamylase (γ -amylase) also may elicit IgE-mediated responses.^{6,7} For industrial use, glucoamylase is usually obtained from *A. niger*, but the enzyme is expressed and secreted by other filamentous fungi, including *Neurospora*.⁸ Although exposure to *Neurospora* spores may induce allergic symptoms and asthma, no reports exist of allergies caused by *Neurospora*-derived enzymes. However, *Neurospora* glucoamylase is 54% identical and 69% similar to *A. oryzae* glucoamylase, and both of the enzymes contain a C-terminal carbohydrate binding module, family 20, domain.³

In summary, we have identified glucoamylase I precursor in *N. crassa* CM using human scFv antibodies. Glucoamylase I precursor is recognized by IgE from some individuals with bakers' asthma and mold allergy, and its presence in vaccines expressed in *N. crassa* needs to be controlled. Besides glucoamylase, antibody NC65 recognizes α -amylase from *A. oryzae* and possibly *S. cerevisiae*, and may be useful as a tool for allergen detection.

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Supplementary Data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.anai.2012.11.021>

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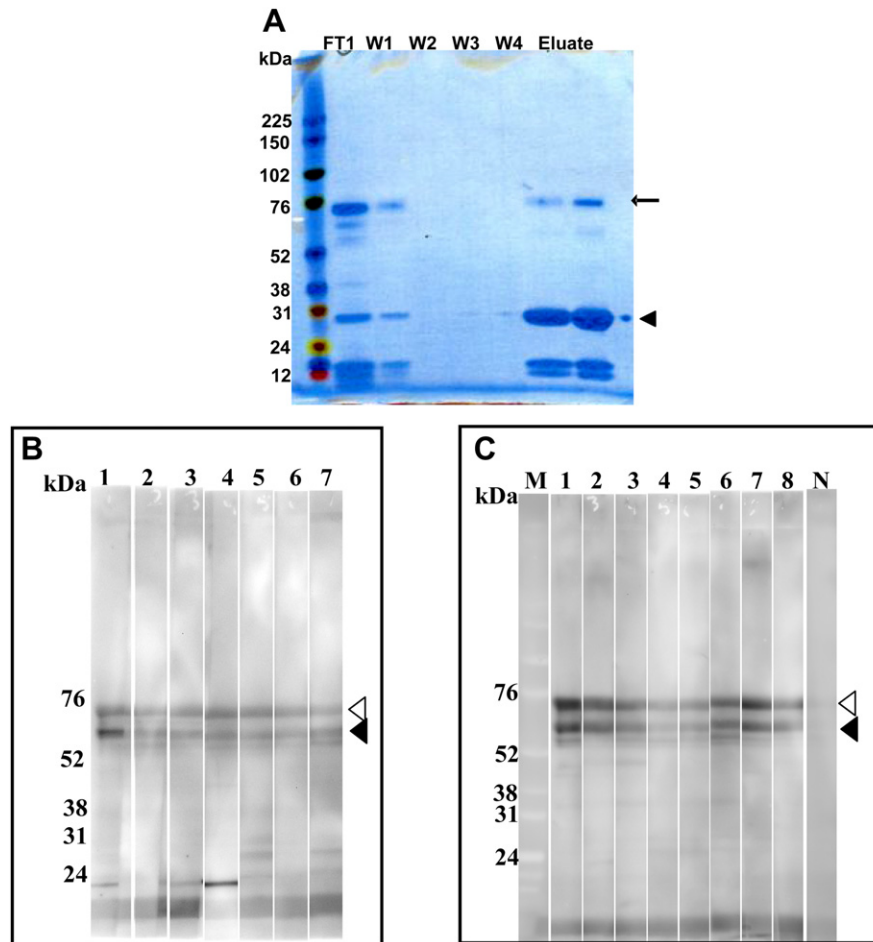


Figure 1. A, Immunoprecipitation of NC65 binding protein from *N. crassa* CM. 4–12%. Bis-Tris gel showing flowthrough (FT1) and washes (w1–w4) collected from the immunoprecipitation column. The *arrow* indicates eluted NC65 target protein that was excised and analyzed, and the *triangle* indicates eluted NC65 antibody. B, IgE immunoblot of CM with sera from bakery workers (lanes 1–7). Biotin-labeled anti-human IgE (1:1,000) and peroxidase-conjugated streptavidin (1:1,000) were used for detection. Open and closed triangles indicate ~70- and 65-kDa bands. C, IgE immunoblot of CM with sera collected from individuals with IgE to *A. fumigatus* (lanes 1–8). N is normal human serum (Thermo Scientific, Rockford, Illinois). Biotin-labeled anti-human IgE (1:1,000) and peroxidase-conjugated streptavidin (1:1,000) were used for detection. *Open* and closed triangles indicate ~70- and 65-kDa bands (See text).

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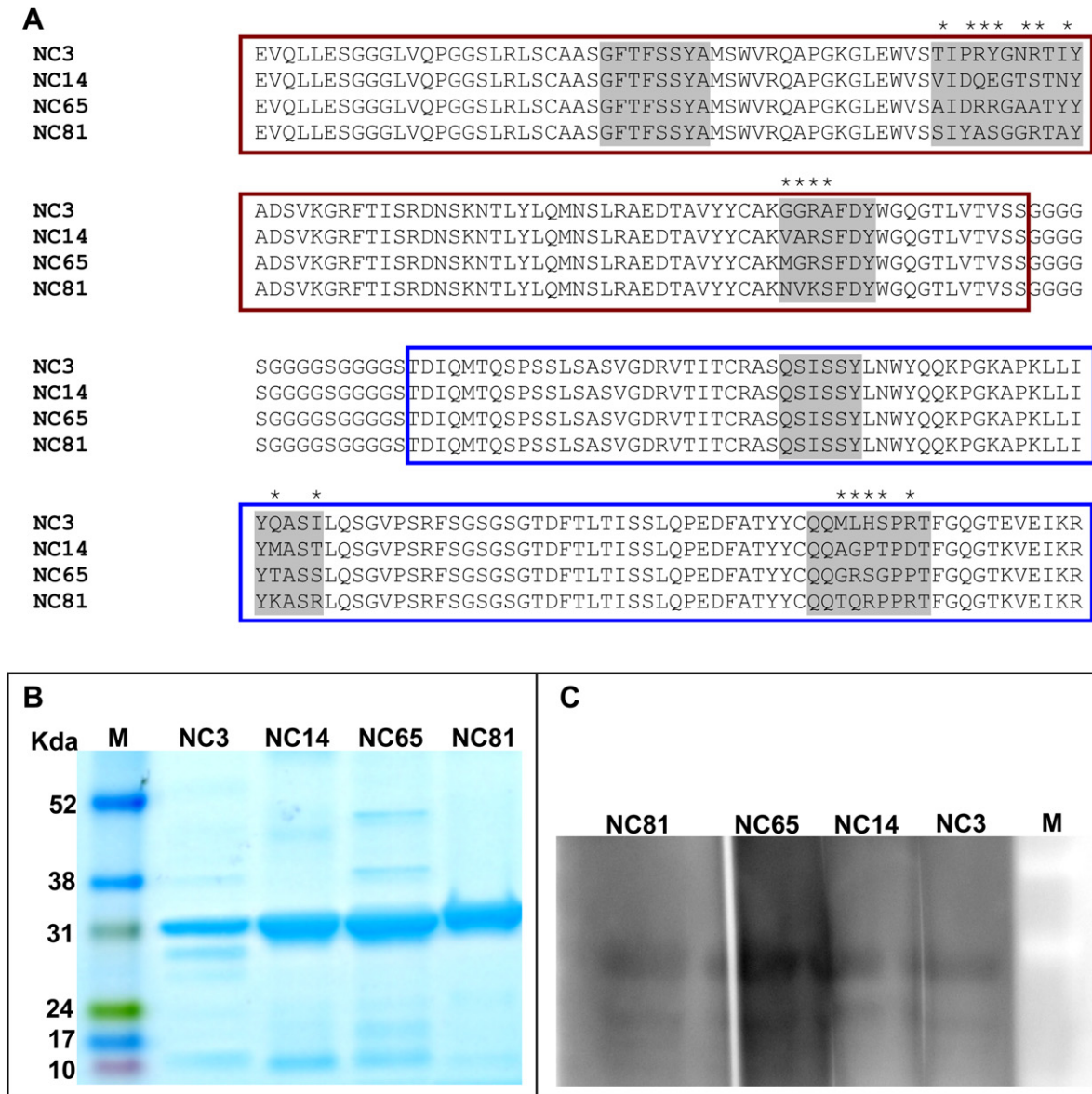
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eFigure 1. A, Comparative sequence data of anti-CM scFv antibodies. V_H domains are in the red box, and the V_L domain is in blue. CDRs are shaded in gray. Asterisks indicate the divergences in the sequence. B, Expressed antibodies were purified by nickel chromatography separated electrophoretically in a 4 to 12% Bis-Tris gel and stained with Coomassie blue. Purified scFv antibodies migrate at 32-kDa molecular weight. C, Immunoblot of *N. crassa* CM using scFv antibodies. After blocking, the polyvinylidene fluoride membrane strips were incubated with 1.0 µg/mL of each indicated scFv antibodies. Horseradish peroxidase-conjugated anti-myc (1:5,000) was used for detection.

A

MHLVSSLLVVGAAFQAVLGLPDLHEKRHSDIIKRSVDSYIQTETPIAQKNLLCNIGASGCRASGAASGV
VVASPSKSSPDYWYTWTRDAALVTKLIVDEFTNDYNTTLQNTIQAYAAAQAKLOGVSNPSGSLSNGAGLG
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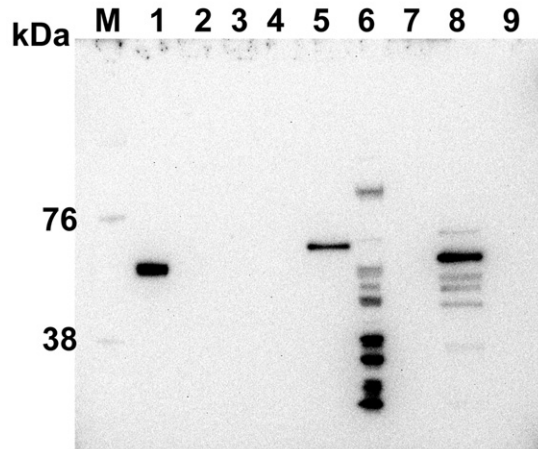
B

Figure 2. A, Amino acid sequence of glucoamylase I precursor protein of *N. crassa*. The peptide sequences identified by MALDI are underlined. The carbohydrate-binding module (CBM20) is in blue. B, Fungal extracts and enzymes were electrophoretically separated and transferred to PVDF membranes. After blocking, the membrane was incubated with NC65. Peroxidase-conjugated anti-myc was used for detection. (1: *A. oryzae* α -amylase; 2: *A. niger* amyloglucosidase; 3: *S. cerevisiae* α -glucosidase; 4: *S. cerevisiae* enolase; 5: *N. crassa* CM; 6: *Neurospora* extract [Greer Laboratories]; 7: *N. crassa* growth medium; 8: yeast extract; 9: *A. fumigatus* extract [Greer Laboratories, Lenoir, North Carolina].)

2.e3