



Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tfac20>

Production and characterisation of monoclonal antibodies for the quantification of potentially allergenic xylanase from *Aspergillus niger*

M. Segal^a, C. Zanetti^a, C. Rizzi^a, M. Olivieri^b, R. Chignola^a & G. Zoccatelli^a

^a Department of Biotechnology, University of Verona, Strada Le Grazie, 15 - CV1, I-37134 Verona, Italy

^b Unit of Occupational Medicine, University Hospital of Verona, c/o Policlinico G. Rossi, I-37134 Verona, Italy

Accepted author version posted online: 31 May 2012. Version of record first published: 24 Jul 2012

To cite this article: M. Segal, C. Zanetti, C. Rizzi, M. Olivieri, R. Chignola & G. Zoccatelli (2012): Production and characterisation of monoclonal antibodies for the quantification of potentially allergenic xylanase from *Aspergillus niger*, Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment, DOI:10.1080/19440049.2012.698657

To link to this article: <http://dx.doi.org/10.1080/19440049.2012.698657>



PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: <http://www.tandfonline.com/page/terms-and-conditions>

This article may be used for research, teaching, and private study purposes. Any substantial or systematic reproduction, redistribution, reselling, loan, sub-licensing, systematic supply, or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae, and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand, or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

Production and characterisation of monoclonal antibodies for the quantification of potentially allergenic xylanase from *Aspergillus niger*

M. Sega^a, C. Zanetti^a, C. Rizzi^a, M. Olivieri^b, R. Chignola^a and G. Zoccatelli^{a*}

^aDepartment of Biotechnology, University of Verona, Strada Le Grazie, 15 – CV1, I-37134 Verona, Italy;

^bUnit of Occupational Medicine, University Hospital of Verona, c/o Policlinico G. Rossi, I-37134 Verona, Italy

(Received 29 February 2012; final version received 25 May 2012)

Xylanase from *Aspergillus niger* (ANX) is widely used in bakeries as a processing aid since it stabilises and improves dough quality. An association between allergic symptoms among bakery workers and sensitisation to ANX has been reported, indicating that this enzyme is an occupational allergen. The presence of ANX in dough improvers and semi-finished goods is often hidden due to incomplete and unclear labelling. The quantification of microbial enzymes in these products is necessary and the determination of the actual concentration of ANX in workplaces is therefore essential to assess the occupational risk. To this purpose we have developed and characterised monoclonal antibodies to ANX. The monoclonal antibodies do not show any cross-reaction with other commonly used microbial enzymes, and they allow the detection of ANX in complex mixtures by ELISA inhibition assays down to the concentration limit of approximately $10 \mu\text{g kg}^{-1}$. These mAbs are a valuable tool to detect and quantify ANX and to investigate its allergenic potential in the workplace.

Keywords: Endo-1,4-beta-xylanase; *Aspergillus niger*; monoclonal antibodies; quantification; wheat flour; dough improvers; occupational allergy

Introduction

Enzymes are commonly added during the processing of several matrices to improve the functional characteristics and/or to standardise the quality of the final products. Cereals represent a prominent target of the enzymes market since important processes such as bread making are fundamentally based on enzyme activity (Poutanen 1997; Sahlstrom and Brathen 1997). For instance, the hydrolysis of starch to fermentable carbohydrates catalysed by amylases is necessary for yeast development and thus bread leavening, but the quality of the final products is also affected by the activity of many enzymes. These can increase dough volume, delay staling, prolong shelf-life and improve organoleptic characteristics (Poutanen 1997; Caballero et al. 2007). Most of the hydrolytic enzymes that catalyse technologically important reactions in cereals are highly expressed in the outer grain layers or in wheat germ, but these components are removed during processing to ameliorate the aspect and shelf-life of baked foods. Such procedures lead to insufficient enzymatic activity in the resulting flours and thus exogenous enzymes are commonly added to target the main components of kernels such as starch, proteins and cell wall polysaccharides during processing.

Recently, attention has been given to pentosans, a group of hemicelluloses also named xylans and more specifically arabinoxylans in cereals. Arabinoxylans represent the major fraction of non-starch polysaccharides and their presence/breakdown has been demonstrated to influence positively some functional properties of doughs such as their viscosity and water-binding capacity, especially when normal bread flour is supplemented with fibre (Butt et al. 2008), as it is commonly done to obtain wholemeal products. Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively acting enzymes. Endo-1,4-beta-xylanases (EC 3.2.1.8) (commonly named “xylanases”) randomly cleave the xylan backbone, beta-D-xylosidases (EC 3.2.1.37) cleave xylose monomers from the non-reducing end of xylo-oligosaccharides and xylobiose, while the removal of the side groups is catalysed by α -L-arabinofuranosidases (EC 3.2.1.55), α -D-glucuronidases (EC 3.2.1.139), acetylxyylan esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and *p*-coumaric acid esterases (EC 3.1.1.-). Complete xylanolytic enzyme sets have been found to be quite widespread among fungi and bacteria (Collins et al. 2005; Polizeli et al. 2005) and it is common practice in the baking industry to use preparations of those

*Corresponding author. Email: gianni.zoccatelli@univr.it

enzymes in order to improve bread quality and dough-handling properties.

In spite of their positive technological role, several microbial enzymes are known to cause allergic reactions in bakery workers, such as baker's asthma and different forms of skin hypersensitivity (Vanhanen et al. 1996; Baur 2005; Harris-Roberts et al. 2009). Previous studies described an association between allergic symptoms among bakery workers and the sensitisation to xylanolytic enzymes (Baur et al. 1998; Sander et al. 1998; Merget et al. 2001; Elms et al. 2003), indicating that these proteins are occupational allergens.

In the European Union the labelling of dough improvers and enriched semi-finished goods frequently does not report microbial enzymes as ingredients; in other cases only the generic name of "food enzymes" is indicated in the labels, without any mention of the source and types of enzymes used. It is therefore difficult to assess the occupational risk for workers exposed to specific enzymes that actually represent hidden occupational allergens. Several antibody-based kits and protocols for the detection of allergens in foodstuffs are commercially available, but to the best of our knowledge no monoclonal antibodies have been developed for the identification and quantification of ANX in food matrices so far.

This paper describes the production and characterisation of mAbs to xylanase from *Aspergillus niger* which is frequently used to improve bread quality (Al-Widyan et al. 2008; Butt et al. 2008). It shows that the mAbs can be used for the quantification of the enzyme dissolved in a complex protein mixture such as dough improvers.

Materials and methods

Enzymes and wheat proteins

This work used different fungal and bacterial enzymes from various commercially available sources. These included enzyme preparations actually in use in the bakery industry. The enzymes were: xylanase (endo-1,4-beta-xylanase M4, E.C. 3.2.1.8) from *Aspergillus niger* (ANX) (Megazyme, Bray, Co. Wicklow, Ireland); xylanase from *A. niger* (Fungal xylanase A, Natural World, Ravenna, Italy); xylanase from *Bacillus subtilis* (Grindamyl Powerbake 900, Danisco, Copenhagen, Denmark); xylanase from *B. subtilis* (Bel'Ase B210, Puratos, Groot-Bijgaarden, Belgium); xylanase from *Thermomyces lanuginosus* (Sigma, Milan, Italy); glucose oxidase from *A. niger* (Frimox B2, Puratos); lipase from *Aspergillus* spp. (Bel'Ase XL1, Puratos); α -amylase from *A. oryzae* (Sigma); pectinase from *A. niger* (Sigma); cellulase from *A. niger* (Sigma); and β -glucanase from *A. niger* (Sigma).

ANX from Megazyme was dialysed against PBS (phosphate-buffered saline) before use. The other enzymes were extracted overnight with PBS (1:10 w/v) at 4°C under continuous shaking. The same procedure was used to extract soluble proteins from wheat flour (cv. Mieti). The insoluble material was separated by centrifugation at 9000 g. Protein concentration was determined by the bicinchoninic acid assay (Pierce, Milan, Italy).

In-gel determination of xylanase activity

Zymogram for xylanolytic activity detection was carried out as previously described (Peterson et al. 2009) with modifications. Protein extracts were separated by 14% SDS-PAGE under reducing conditions. The gel was incubated for 2 h at room temperature with a 2.5% (v/v) Triton X-100 (Sigma) solution to allow protein renaturation and for a further 30 min with 0.1 M sodium acetate buffer pH 5.2. The gel was overlaid with a 7% polyacrylamide gel pre-equilibrated with sodium acetate and co-polymerised with 1.13% (w/v) remazol brilliant blue R-D-xylan (Sigma). After 1-h incubation at 50°C the gel was rinsed with 2:1 (v/v) 70% ethanol and 0.1 M sodium acetate buffer. This treatment destains those areas where the xylan substrate is degraded and thus active xylanolytic enzymes appear as lighter bands on a darker-blue background.

Reduction and alkylation of xylanase

ANX has a rather compact globular structure. It contains three cysteine residues, and two of them form a disulfide bridge (Krengel and Dijkstra 1996). Full reduction of the disulfide bridge was obtained by incubating xylanase (Megazyme) with a solution containing 10 mM DTT (dithiothreitol), 8 M urea, 0.1 M Tris-HCl pH 8 for 1 h at room temperature. To avoid spontaneous re-oxidation of cysteines both in solution and in gel, the reduced enzyme was further treated with 50 mM iodoacetamide (IAA) for 1 h in the dark at room temperature. Residual IAA was then blocked with an equimolar concentration of DTT. Reduced and alkylated (R/A) xylanase was dialysed overnight at 4°C with PBS. A batch of xylanase was also treated with IAA as previously described without reduction of the disulfide bonds as a control.

Mice immunisation and production of monoclonal antibodies (mAbs)

Mice were immunised with ANX (Megazyme) as described in the Supplementary Material (supplementary material is available on the article webpage). Experiments were carried out according to current

Italian bioethic laws (D.L. 116, 1992) in agreement with the directions mandated by 86/609/CEE on animal experimentation. To obtain immortalised mAbs-secreting hybridomas, mice were sacrificed and spleen cells were incubated at a 5:1 ratio with Ag8 cells (murine myeloma) in the presence of 50% (w/v) polyethylene glycol (PEG) 1500 (Chignola et al. 2000). Hybridomas were grown in RPMI 1640 medium and selected by progressively replacing RPMI with HAT (hypoxanthine aminopterin thymidine) minimal medium (HAT media supplement (50×) Hybri-MaxTM, Sigma). After 6 weeks cells were cloned at 0.3 cells/well in 96-well culture plates filled with HAT medium supplemented with 20% foetal bovine serum (FBS). Supernatants were then screened for antibody production by ELISA using 96-well microtitre plates (Maxisorp, Nunc A/S, Roskilde, Denmark) coated with 2 µg/well ANX (Megazyme) (see the “Supplementary Material”). Non-specific binding sites were saturated with a solution containing 1% BSA (w/v) in PBS and 0.05% (v/v) Tween-20 (PTB buffer), and bound antibodies revealed with either horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Immunoglobulin G) or anti-mouse poly-Ig antibodies (Sigma) diluted 1:1000 in PTB. The detection was achieved by TMB solution (3,3',5,5'-tetramethyl benzidine; Sigma), using a Powerwave microplate reader (Bio-Tek). Positive clones were then expanded in T-25 flasks and supernatants from stable clones were collected, filter-sterilised and stored at 4°C.

Isotyping and quantification of mAbs

The isotype of selected antibodies was determined using commercially available kits following the manufacturer's procedures (IsoStrip Mouse Monoclonal Antibody Isotyping Kit, Roche Diagnostic GmbH, Mannheim, Germany).

The quantification of IgG mAbs in stored culture supernatants was carried out by ELISA using known amounts of standard murine IgG antibodies (Mouse Immunoglobulin Isotype Panel, Southern Biotech, Birmingham, AL, USA) for appropriate reference. Microtitre plates were coated with goat anti-mouse IgG (Southern Biotech) at a 1:200 dilution in PBS (determined in preliminary independent assays) and then saturated with PTB. Antibody-containing supernatants or standard IgG antibodies were added at 1:2 serial dilutions in PTB (starting from 0.02 µg µl⁻¹) and captured antibodies were then measured using HRP-conjugated goat anti-mouse IgG antibodies (Sigma), diluted 1:1000 in PTB. The supernatants from non-Ig-producing cell clones were used as a negative control. The detection was carried out as described above.

Determination of mAbs binding affinity

To measure the binding constants of selected mAbs we followed the method described by High et al. (2005). The method allows mAbs at a fixed initial concentration ($[Ab]_0$, where square brackets denote molar concentrations) to bind various initial concentrations of antigen ($[Ag]_0$) in solution in 96-well microtitre plates saturated overnight with PTB to avoid the absorption of both antibodies and antigen. Free antibodies ($[Ab]$) are then measured by standard ELISA assays on microtitre plates coated with a fixed amount of antigen, and optical density (OD) measurements converted into concentration units using a standard curve obtained in parallel with known amounts of mAbs. Since at equilibrium:

$$[Ab]_0 = [Ab] + [AbAg]$$

$$[Ag]_0 = [Ag] + [AbAg]$$

both bound antibody/antigen and free antigen concentrations ($[AbAg]$ and $[Ag]$, respectively) can be calculated straightforwardly. The equilibrium dissociation constant (K_D) was then determined by fitting data in a “bound versus free” plot with the following equation:

$$[AbAg] = \frac{[Ab]_0[Ag]}{K_D + [Ag]} \quad (1)$$

In the assays, PTB-blocked plates containing mAbs only in the concentration range 0.002–0.675 µg ml⁻¹ were incubated overnight, and these samples were then measured by ELISA assays carried out in plates coated with 2 µg/well ANX (Megazyme). Data were then fitted with a five-parameter logistic equation (standard curve). In parallel, we allowed 0.04 µg ml⁻¹ of mAbs to bind overnight at room temperature to variable ANX concentrations (in the range of 0.009–2.7 µg ml⁻¹) in PTB-blocked ELISA plates. Free antibody concentrations were then measured in ELISA assays carried out with xylanase-coated wells (2 µg/well) and OD units were converted into concentration units by backward extrapolation of the data onto the standard curve.

The molar concentration of the mAbs was calculated assuming a valence of 2 and a molecular weight of 147 kDa for IgG antibodies.

All the samples were analysed in triplicate and both standard deviations (SD) and the propagation of the errors (due to the backward extrapolations) were calculated. The software Mathematica (Wolfram Research Inc., v. 8.0) was used to implement the mathematical procedures and compute all the calculations.

Western blotting

Proteins separated by SDS-PAGE were electroblotted onto polyvinylidene fluoride membranes

(PVDF; Millipore, Milan, Italy). Membranes were then probed overnight at room temperature with cultured clones supernatants diluted 1:10 in blocking solution (3% w/v skimmed dry milk and 0.05% Tween-20 in PBS). Bound mAbs were detected using alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Sigma) diluted 1:2000 in blocking solution. The detection was achieved by BCIP/NBT (nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyl-phosphate) substrate (Bio-Rad, Munich, Germany). As a positive control, polyclonal antibodies from the sera of immunised animals were used, whereas as a negative control we employed complete culture medium.

Biotinylation of mAbs

The cross-reactivity of mAbs was analysed in competition ELISA assays. To this purpose, mAbs from one selected clone (P5B8) were biotinylated as follows: 2.5 ml of culture supernatants (containing approximately 12 mg of total protein) were equilibrated in 50 mM sodium carbonate buffer pH 8 by gel filtration onto PD10 columns (GE Healthcare Biosciences, Uppsala, Sweden). Biotinamidocaproate N-hydroxy-succinimidyl ester (Sigma) (1.68 mg in dimethyl sulfoxide) was then added to obtain a 5:1 biotin:total protein molar ratio (considering FBS albumin as the major component) and the sample was incubated for 2 h at room temperature under shaking. The reaction was stopped adding 134 μ l of a 1 M ammonium chloride solution followed by overnight dialysis with PBS at 4°C.

Competition ELISA for epitope analysis

Competition ELISA assays were performed on microtitre plates coated with 2 μ g/well ANX (Megazyme). Three-fold serial dilutions of unlabelled mAbs were prepared in the presence of a fixed concentration of biotinylated P5B8 mAb (0.08 μ g ml⁻¹) and used to probe the coated xylanase. The binding of unlabelled

mAbs was revealed using HRP-conjugated goat anti-mouse IgG antibodies (1:1000 dilution in PTB; see above), whereas 0.1 μ g ml⁻¹ HRP-streptavidin (Sigma) dissolved in PTB was used to detect biotinylated P5B8 mAb.

ELISA inhibition for xylanase quantification

Ten-fold serial dilutions of ANX (Megazyme) were prepared in 2 mg ml⁻¹ wheat protein extract and P3F10 mAb was then added at the final 1:500 dilution. The reaction was allowed to proceed at room temperature for 1 h, and the binding activity of free P3F10 antibodies assayed in standard ELISA assays on ANX-coated plates as described above.

Results

Production of anti-xylanase mAbs

In spite of the successful immunisation of animals, the selection and further growth of mAbs-secreting hybridomas were rather difficult (see the Supplementary Material and the Discussion). After cloning, the frequency of growing clones was much lower than expected and none of the antibody-producing clones was stable except five out of 11 highly secreting hybridomas, which were selected and further propagated. All mAbs from these five cell clones (named P3F10, P1D2, P5B8, P2C2 and P5D5) were of the Ig(G1, κ) isotype, but the efficiency of their secretion by cells in culture supernatants varied among isolated clones (Table 1). mAbs' binding affinity to xylanase was measured by ELISA following the procedure described in the Materials and methods (Figure 1); the results are given in Table 1.

Epitopes analysis

ANX was digested with various proteolytic enzymes (pepsin, papain, trypsin and chymotrypsin) using different enzyme:protein ratios (up to a 300:1 molar

Table 1. Biochemical characteristics of selected mAbs.

Clone	Isotype	Concentration (μ g ml ⁻¹) ^a	K_D (nM) ^b	P^c	r^2 ^d
P3F10	IgG1, κ	25.4	1.51 ± 0.14	8.6×10^{-8}	0.99
P1D2	IgG1, κ	13.4	1.09 ± 0.16	7.8×10^{-6}	0.98
P5B8	IgG1, κ	12.3	1.35 ± 0.11	2.6×10^{-8}	0.99
P2C2	IgG1, κ	5.2	1.98 ± 0.08	2.5×10^{-13}	0.99
P5D5	IgG1, κ	8.9	1.95 ± 0.15	1.0×10^{-8}	0.99

Notes: ^aThe concentration of mAbs was measured in culture supernatants by ELISA as described in the Materials and methods section at comparable cell density.

^bThe equilibrium dissociation constant was estimated by fitting experimental data with equation (1).

^cProbability values for parameter t -statistics.

^dGoodness-of-fit statistics.

ratio). However, xylanase showed strong resistance to proteolytic attack (data not shown). The digestion was only successful after denaturation and reduction of the disulfide bond followed by an alkylation step, and this in turn suggests that the protein has a stable disulphide bond. The five selected mAbs could not bind the digestion products (data not shown). Indeed, all the selected mAbs could recognise the xylanase treated separately with urea, DTT and IAA in a Western blot, but not the R/A protein (Figure 2), suggesting that the immune epitope is linear and involves an amino acid sequence that includes one of the two disulfide bond-forming cysteines.

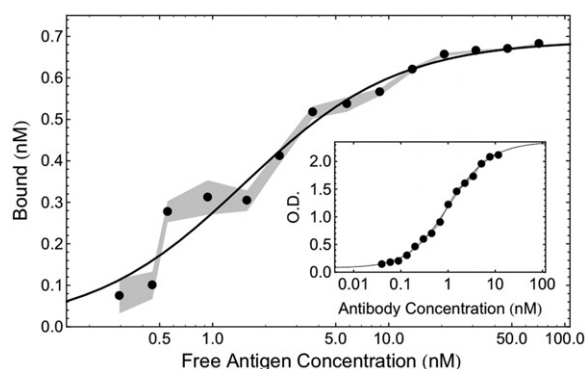


Figure 1. Measuring the affinity of mAbs/xylanase binding by ELISA. A representative result is shown. The affinity was measured at equilibrium in solution following the method described by High et al. (2005). The black line shows the best fit to experimental data with equation (1). Inset: plot of the standard curve obtained with known concentrations of mAb and used to extrapolate the concentration of free antibodies in the binding reaction from OD data. Experimental data were fitted with a five-parameter logistic equation. In both panels the grey shaded area around points show the standard deviation (SD). In the main panel SD values are higher because of the propagation of errors through calculations.

Since all mAbs showed the same binding pattern (Figure 2), we set out to develop a competition ELISA assay to study whether immune epitopes bound by mAbs could overlap. To this purpose, we labelled one mAb (P5B8) with biotin and used it at a fixed concentration in standard ELISA assay on ANX-coated plates. All mAbs could inhibit P5B8 binding to xylanase (Figure 3), indicating that the immune epitopes recognised by mAbs comprise overlapping amino acid sequences on the antigen.

Binding specificity of P3F10 mAb

Data given above show that the mAbs could not be used to develop sandwich ELISA assays. We therefore decided to put forward this study for one mAb only, and P3F10 was chosen because of its highest production by cells and its relative high affinity (Table 1).

Figure 4(a) shows a representative Coomassie-stained SDS-PAGE separation of several enzymes and enzymatic preparations used in bakeries. Figure 4(b) shows the corresponding zymogram. Notably, some samples (lanes 2–5 and 9–11) display xylanolytic activity bands with different mobility in comparison with ANX, indicating that they are capable of hydrolysing xylan and/or they are contaminated by xylanases from other origins. These proteins are not recognised by P3F10 mAb that, in fact, binds xylanase from *A. niger* only (Figure 4c).

Quantification of xylanase from *A. niger* in a complex mixture

In order to detect xylanase dissolved in a complex mixture such as a wheat salt-soluble protein extract, we set out to develop ELISA inhibition experiments, as previously described (Zoccatelli et al. 2010; Consolini et al. 2012). The results are shown in Figure 5. The detection of free mAb molecules saturated at the corresponding xylanase concentration of 1 ng ml^{-1} ,

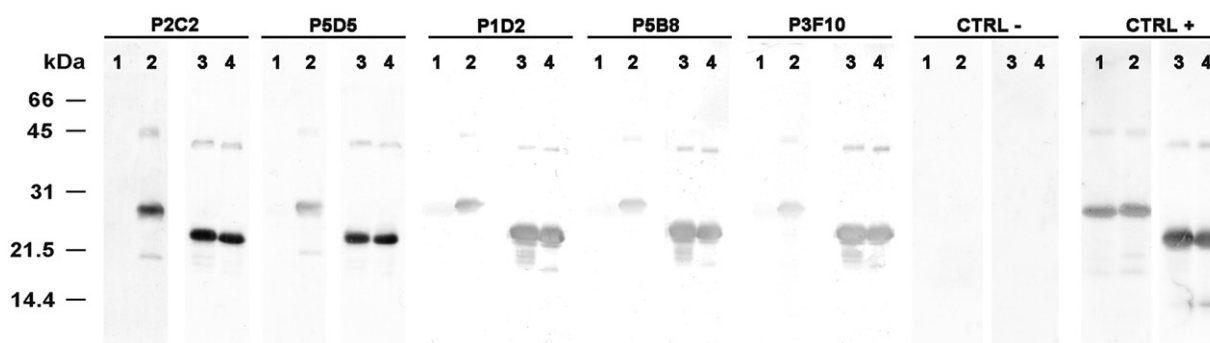


Figure 2. SDS-PAGE and immunoblotting for assaying the binding of mAbs as function of reduction and alkylation. 1, R/A xylanase; and 2–4, xylanase treated, respectively, with 10 mM DTT (2), 50 mM IAA (3) and 8 M urea (4). Samples were immunodetected with the different mAbs, the serum of immunised mice (CTRL +) and the culture medium (CTRL –).

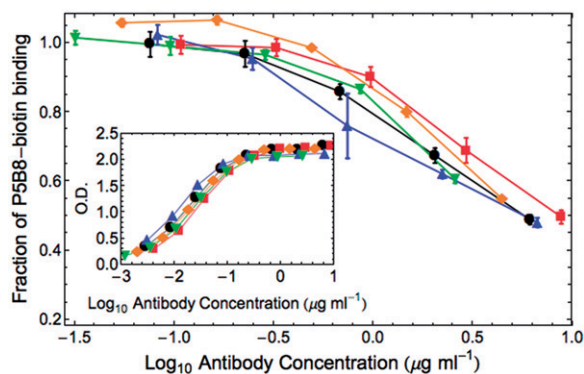


Figure 3. Competition ELISA assays. P5B8 mAb was labelled with biotin and used at a fixed concentration on xylanase-coated ELISA plates. The fraction of residual P5B8-biotin binding activity is shown as a function of increasing concentrations of unlabelled mAbs. Inset: binding of mAbs to xylanase in standard ELISA assays. In both panels, colours indicate the results obtained with the following unlabelled mAbs: black (circles), P5B8; red (squares), P3F10; blue (triangles up), P1D2; green (triangles down), P2C2; and orange (diamonds), P5D5.

and this means that all antibody molecules in solution were free to bind xylanase-coated wells or, conversely, that xylanase at this concentration could not significantly trap antibody molecules in solution. Thus, the limit of detection (LOD) of this method was approximately 1 ng ml^{-1} and the limit of quantification (LOQ) was calculated to be 10 ng ml^{-1} . Since in the protocols wheat flour was extracted at a 1:10 ratio, the threshold of the assay could also be established as being approximately $10 \mu\text{g kg}^{-1}$.

Discussion

The exposure to flour dust is well known to induce occupational allergies, and in particular asthma, in bakery workers (Walsh et al. 1985; Baur et al. 1988; Gomez et al. 1990; Baur and Posch 1998). There is evidence that subjects affected by this pathology are sensitised to microbial enzymes used as dough improvers, among which are xylanase preparations from *A. niger* (Baur et al. 1998; Sander et al. 1998; Merget et al. 2001; Elms et al. 2003). Unclear labelling is a further issue; indeed, producers often do not provide reliable information on protein amounts in enzyme preparations or any information at all, and this in spite of the fact that the European Commission has recently adopted a regulation which introduces harmonised legislation on food enzymes. This means that food enzymes actually represent hidden potential allergens for workers. Knowing the actual concentration of these allergens in workplaces is essential to determine the occupational risk for bakers, especially when the proteins are not clearly declared in the labels. Methods based on mAbs are therefore warranted specifically to

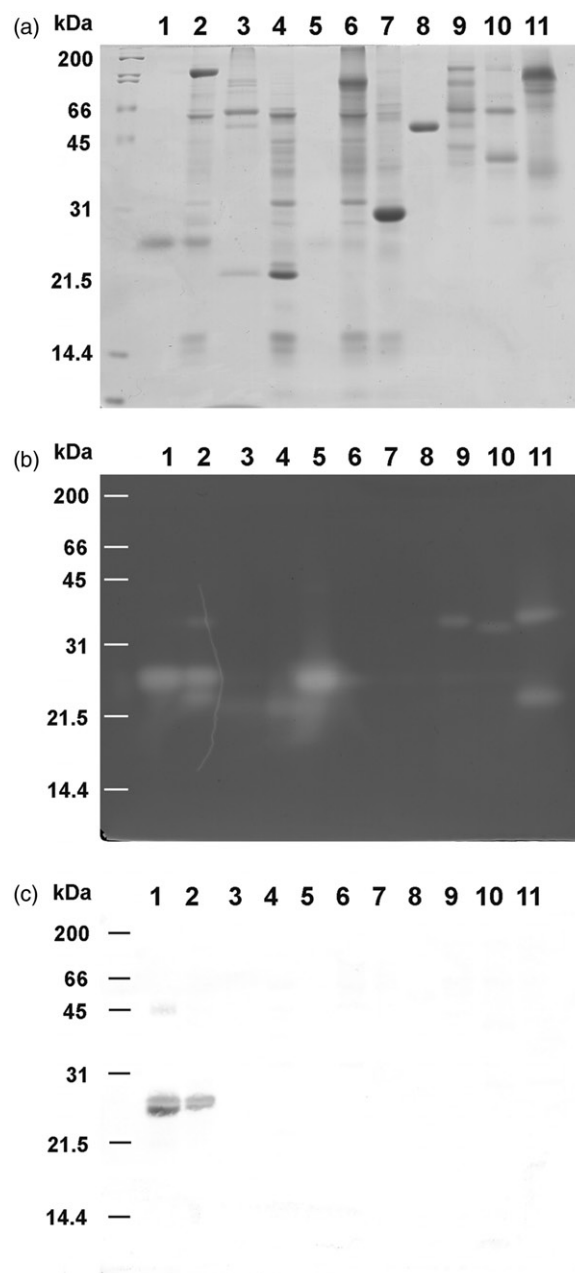


Figure 4. SDS-PAGE under reducing conditions of the samples of enzymes and enzyme preparations used in the present work. (a), Coomassie staining; (b), zymogram with remazol brilliant blue R-D-xylan; (c), immunoblotting with P3F10 mAb; 1, xylanase from *A. niger* (ANX) (Megazyme); 2, xylanase from *A. niger* (Natural World); 3, xylanase from *B. subtilis* (Danisco); 4, xylanase from *B. subtilis* (Puratos); 5, xylanase from *Thermomyces lanuginosus* (Sigma); 6, glucose oxidase from *A. niger* (Puratos); 7, lipase from *Aspergillus* spp. (Puratos); 8, α -amylase from *A. oryzae* (Sigma); 9, pectinase from *A. niger* (Sigma); 10, cellulase from *A. niger* (Sigma); and 11, β -glucanase from *A. niger* (Sigma).

detect and quantify allergens in workplaces, and thus to establish concentration limits of exposure for workers.

We immunised mice using conventional protocols that in general lead to a potent secondary humoral

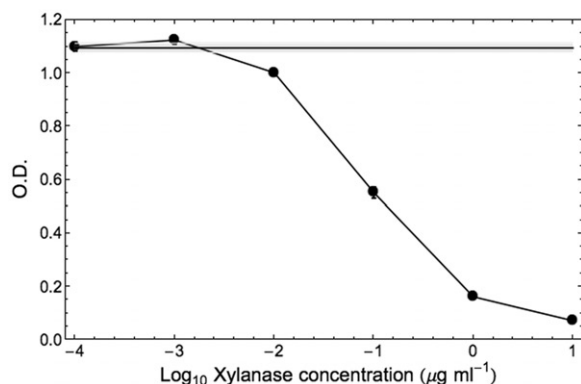


Figure 5. Quantification of xylanase from *A. niger* in wheat extracts by ELISA inhibition. The enzyme was dissolved at the indicated concentrations in the presence of 2 mg ml⁻¹ wheat protein extracts. A fixed concentration of P3F10 mAb was then added to the solution. Free P3F10 mAb molecules were then measured by standard ELISA on xylanase-coated plates as described in the text. As expected, high xylanase concentrations in wheat protein extracts trap antibody molecules in solution and inhibit their binding to xylanase-coated plates. The continuous black line on top and the grey shaded area around it show the mean $\pm$ standard deviation (SD) of P3F10 mAb binding to xylanase-coated plates as measured by standard ELISA; they are plotted for appropriate reference as positive control.

immune response. This response is well known to be characterised by a dominant proportion of high-affinity IgG antibodies, thus increasing the probability to isolate mAbs to be used for detection/quantification purposes. This apparently is not the case for *A. niger* xylanase that, in both immunisations, stimulated a higher proportion of antibodies belonging to classes other than the G one. Many of these mAbs could be tested before cells were lost and resulted to be low-affinity IgM (i.e. we could not measure K_D using the method described in the Materials and methods section). In addition, all the five selected clones appeared to bind by overlapping linear epitopes on ANX comprising one of the two cysteine residues forming the disulfide bond.

It is not the aim of the present work to discuss these results further, but the authors nonetheless wish to point out that the immunologic properties of this enzyme are interesting enough to deserve further investigations.

We characterised the specificity of P3F10 mAb and we show that the antibody selectively binds ANX. By using P3F10 mAb we developed an ELISA inhibition method that allows one to reach an LOD of approximately 10 µg kg⁻¹ and an LOQ of 100 µg kg⁻¹.

Data reported in the literature indicate that in bakery practice the amount of added improver is about 1–5% of product (Merget et al. 2001). However, manufacturers frequently suggest lower doses (about 1–10 g per 100 kg of flour). As an example,

the manufacturer's recommended dosage for "Fungal xylanase A" improver (Natural World) is 3–5 g per 100 kg of flour. The improver is not pure, and we estimated by ELISA a xylanase content of 9.53 g kg⁻¹ that, according to recommended dosages, results in a final ANX content of 286–476 µg kg⁻¹. Both LOQ and LOD of the method described in this work are below the suggested dosage range. This shows that the mAbs can actually be used for the detection and quantification of *A. niger* xylanase in wheat flours and improvers.

The use of the mAbs could be extended to other industrial fields in which *A. niger* xylanase is largely employed, e.g. paper manufacturing, textile, animal feed, and the juice and wine industries.

Acknowledgements

This work was financed by grants from the Fondazione Cariverona (Project Title: 'Development of Study Models for Safety Assessment of Enzymes Used in Cereal Flours'), Istituto Nazionale Assicurazione contro gli Infortuni sul Lavoro (INAIL) and Novartis.

References

- Al-Widyan O, Khataibeh MH, bu-Alruz K. 2008. The use of xylanases from different microbial origin in bread baking and their effects on bread qualities. *J Appl Sci.* 8(4):672–676.
- Baur X. 2005. Enzymes as occupational and environmental respiratory sensitizers. *Int Arch Occup Environ Health.* 78(4):279–286.
- Baur X, Posch A. 1998. Characterized allergens causing bakers' asthma. *Allergy.* 53(6):562–566.
- Baur X, Sander I, Posch A, Raulf-Heimsoth M. 1998. Baker's asthma due to the enzyme xylanase – a new occupational allergen. *Clin Exp Allergy.* 28(12):1591–1593.
- Baur X, Sauer W, Weiss W. 1988. Baking additives as new allergens in baker's asthma. *Respiration.* 54(1):70–72.
- Butt MS, Tahir-Nadeem M, Ahmad Z, Sultan MT. 2008. Xylanases and their applications in baking industry. *Food Technol Biotechnol.* 46(1):22–31.
- Caballero PA, Gomez M, Rosell CM. 2007. Improvement of dough rheology, bread quality and bread shelf-life by enzymes combination. *J Food Eng.* 81(1):42–53.
- Chignola R, Cestari T, Guerriero C, Riviera AP, Ferrari S, Brendolan A, Gobbo M, Amato S, Sartoris S, Fracasso G, et al. 2000. Expression of myelin basic protein (MBP) epitopes in human non-neural cells revealed by two anti-MBP IgM monoclonal antibodies. *Clin Exp Immunol.* 122(3):429–436.
- Collins T, Gerday C, Feller G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol Rev.* 29(1):3–23.
- Consolini M, Segà M, Zanetti C, Fusi M, Chignola R, De Carli M, Rizzi C, Zoccatelli G. 2012. Emulsification of simulated gastric fluids protects wheat alpha-amylase

- inhibitor 0.19 epitopes from digestion. Food Anal Method. 5(2):234–243.
- Elms J, Fishwick D, Walker J, Rawbone R, Jeffrey P, Griffin P, Gibson M, Curran AD. 2003. Prevalence of sensitisation to cellulase and xylanase in bakery workers. Occup Environ Med. 60(10):802–804.
- Gomez L, Martin E, Hernandez D, Sanchez-Monge R, Barber D, del Pozo V, de Andrés B, Armentia A, Lahoz C, Salcedo G. 1990. Members of the alpha-amylase inhibitors family from wheat endosperm are major allergens associated with baker's asthma. FEBS Lett. 261(1):85–88.
- Harris-Roberts J, Robinson E, Waterhouse JC, Billings CG, Proctor AR, Stocks-Greaves M, Rahman S, Evans G, Garrod A, Curran AD, et al. 2009. Sensitization to wheat flour and enzymes and associated respiratory symptoms in British bakers. Am J Ind Med. 52(2):133–140.
- High K, Meng Y, Washabaugh MW, Zhao Q. 2005. Determination of picomolar equilibrium dissociation constants in solution by enzyme-linked immunosorbent assay with fluorescence detection. Anal Biochem. 347(1):159–161.
- Krengel U, Dijkstra BW. 1996. Three-dimensional structure of endo-1,4-beta-xylanase I from *Aspergillus niger*: molecular basis for its low pH optimum. J Mol Biol. 263(1):70–78.
- Merget R, Sander I, Raulf-Heimsoth M, Baur X. 2001. Baker's asthma due to xylanase and cellulase without sensitization to alpha-amylase and only weak sensitization to flour. Int Arch Allergy Immunol. 124(4):502–505.
- Peterson R, Grinyer J, Joss J, Khan A, Nevalainen H. 2009. Fungal proteins with mannanase activity identified directly from a Congo Red stained zymogram by mass spectrometry. J Microbiol Methods. 79(3):374–377.
- Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol. 67(5):577–591.
- Poutanen K. 1997. Enzymes: an important tool in the improvement of the quality of cereal foods. Trends Food Sci Technol. 8(9):300–306.
- Sahlstrom S, Brathen E. 1997. Effects of enzyme preparations for baking, mixing time and resting time on bread quality and bread staling. Food Chem. 58(1–2):75–80.
- Sander I, Raulf-Heimsoth M, Siethoff C, Lohaus C, Meyer HE, Baur X. 1998. Allergy to *Aspergillus*-derived enzymes in the baking industry: identification of beta-xylosidase from *Aspergillus niger* as a new allergen (Asp n 14). J Allergy Clin Immunol. 102(2):256–264.
- Vanhanen M, Tuomi T, Hokkanen H, Tupasela O, Tuomainen A, Holmberg PC, Leisola M, Nordman H. 1996. Enzyme exposure and enzyme sensitisation in the baking industry. Occup Environ Med. 53(10):670–676.
- Walsh BJ, Wrigley CW, Musk AW, Baldo BA. 1985. A comparison of the binding of IgE in the sera of patients with bakers' asthma to soluble and insoluble wheat-grain proteins. J Allergy Clin Immunol. 76(1):23–28.
- Zoccatelli G, Pokoj S, Foetisch K, Bartra J, Valero A, Del Mar San Miguel-Moncin M, Vieths S, Scheurer S. 2010. Identification and characterization of the major allergen of green bean (*Phaseolus vulgaris*) as a non-specific lipid transfer protein (Pha v 3). Mol Immunol. 47(7–8):1561–1568.