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Purification of a new fungal mannose-specific lectin from *Penicillium chrysogenum* and its aphicidal properties

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ABSTRACT

Several Ascomycetes fungi are commonly used in bio-industries and provide available industrial residues for lectin extraction to be valuable. A lectin from *Penicillium chrysogenum*, named PeCL, was purified from a fungal culture using gel-filtration chromatography column. PeCL was found to be a mannose-specific lectin by haemagglutination activity towards rabbit erythrocyte cells and was visualised on SDS-PAGE gel. Purified PeCL fraction was delivered via artificial diet to *Myzus persicae* aphid and was demonstrated to be aphicidal at 0.1 % with higher toxic efficiency than a known mannose-binding lectin Concanavalin A (ConA). A fast and efficient way to purify PeCL and a potential use in pest control is described.

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Introduction

Lectins are carbohydrate-binding proteins that are widely distributed in nature and play different roles and functions in biological processes such as recognition molecules within the immune system in animals (Kilpatrick 2002) and as storage proteins or in defence mechanisms against pest and pathogens in plants (Peumans & Van Damme 1995; Van Damme *et al.* 1998; Rudiger & Gabius 2001). Lectins have also been purified from fungi and were found to be involved in host recognition, cell surface adhesion, antiproliferative action and endocytosis (Guillot & Kanska 1997; Francis *et al.* 2003; Ng 2004; Wang *et al.* 2006). In particular, the use of endophytic fungi producing lectin was

found to be a promising way to deliver insecticidal proteins to control sap-sucking pests for crop protection (Qi *et al.* 2011). By example, *Sclerotinia sclerotiorum* agglutinin induced a high mortality on *Acyrtosiphon pisum* fed with fungal lectin in artificial diet (Hamshou *et al.* 2010). Only a few lectins have been extracted from Ascomycetes (Jung *et al.* 2007) even if some of these fungi are very common in bio-industries. Among them, *Penicillium chrysogenum* was selected here due to its very common availability and the potential use of industrial residues as a source of potential bioinsecticide proteins. Indeed, the ability of lectins to interact with carbohydrates and combine with glycol-components of cell surface has been investigated to find toxic proteins to control insect pests as alternative methods

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to conventional chemical control (Macedo et al. 2007). A range of Hemiptera including aphids cause serious damage to many crop plants by directly sucking plant sap and by indirectly acting as virus vectors (Barton et al. 1987; Peferoen et al. 1990).

Lectins have obvious potential as insect control agents although knowledge as to the mechanisms of lectin action is limited. Insecticidal lectins have been shown to be bound to midgut epithelial cells in a variety of pest species (Habibi et al. 1998, 2000). In some cases, damage to structure of gut epithelium, such as that observed by Powell et al. (1998) in the Hemiptera *Nilaparvata lugens* has led to suggestions that binding may be a causative factor in the toxicity of a wide range of lectins to insects. The sensitivity of different insect species to the effects of lectin ingestion is variable, and binding of a particular lectin to the gut of a given species does not necessarily facilitate insecticidal activity (Harper et al. 1995). The blockage of the peritrophic matrix pores by lectins was at least in part responsible for observed insect starvation effects. Bioassays with Con A corresponded to the highest observed toxicity among the tested lectins towards several insect species (Fitches et al. 2001) and lead to the selection of Concanavalin A (ConA) as referenced insecticidal lectin in further assays. Some previous studies have been conducted which have shown mannose-binding plant lectins with antimetabolic properties towards Hemiptera insect pests including aphids, planthoppers, and leafhoppers (Sauvion et al. 1996; Powell 2001). However, relatively few studies have focused on the potential use of fungal-derived lectins to achieve this aim. Here, a lectin from *P. chrysogenum* was purified in a simple protocol based on gel-filtration chromatographic technique. The characterisation of the lectin was performed by haemagglutination assays in the presence of various carbohydrates and by electrophoresis. Finally, the toxicological effect of the purified lectin was determined on a single aphid species *Myzus persicae* Sultzer. The potential use of this fungal lectin as myco-insecticide is discussed in relation to the development of biological control molecules to target major crop pests.

Materials and methods

Fungal growth and purification procedure

Penicillium chrysogenum was cultured in 863 medium (peptone 10 g, glucose 20 g, and yeast extract 10 g) in 300 ml Erlenmeyer flasks with a 200 ml working volume on a rotary shaker (150 rpm) at 30 °C for 96 h. The culture broth was filtered through a vacuum pump and mycelium was collected. Forty grams of harvested mycelia were ground into powder in liquid nitrogen. Proteins from the mycelium powder were extracted in phosphate buffered saline (PBS: 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl, pH, 7.4) and the homogenate was filtered through cheesecloth before being centrifuged at 15 000 g for 20 min at 4 °C. The supernatant was collected and concentrated 10 times by ultrafiltration (3000 Da) at 4000×g and 4 °C and applied to a prepacked column 16/70 connected with a FPLC Äkta (GE Healthcare, Diegem, Belgium). Proteins were eluted with 150 ml of PBS pH 7.4, the flow was fixed at 0.2 ml min⁻¹. Fractions of 4 ml were collected. The column has been calibrated with a GE Healthcare molecular weight

marker kit (Ovalbumin 43 000 Da, Carbonic anhydrase 29 000 Da, Ribonuclease A 13 700 Da, and Aprotinin 6500 Da). Fraction size: 4 ml; injection at 'Elution = 0 ml'; flow rate: 0.2 ml min⁻¹; absorbance at 280 nm.

Haemagglutination activity and inhibition assays

Rabbit erythrocytes were used for the determination of haemagglutination activity. The erythrocyte cells were washed (three times) in PBS (4 % v v⁻¹, pH 7.4), centrifuged (2000×g, for 15 min at 4 °C), and resuspended in PBS. For haemagglutination assays, 50 µl of two-fold serial dilutions of the protein extracts were mixed with an equal volume of a 4 % erythrocyte suspension in wells of U-shaped microtiter plates. The plates were allowed to settle at room temperature for 2 h when the negative control had fully sedimented.

Inhibition assays using carbohydrates were performed by serial two-fold dilutions of specific sugars in PBS. The tested carbohydrates were D-fructose, D-galactose, D-glucose, D-mannose, Sucrose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and L-fructose. Each dilution was mixed with an equal volume (25 µl) of protein sample and allowed to stand for 30 min at 4 °C before mixing with 50 µl of 4 % rabbit erythrocyte suspension. The haemagglutination activity was conducted as described above. The minimum concentration of each tested sugar in the final reaction mixture allowing complete inhibition of haemagglutination units was recorded.

Protein concentration determination

Protein concentrations were determined according to the Bradford method with bovine serum albumin as the standard (Bradford 1976).

Protein denaturation

For analytical SDS/PAGE, samples were diluted 1:4 with a solubilizer (1 % SDS; 0.02 % bromophenol; 1 % beta-mercaptoethanol in running buffer) and boiled for 3 min before electrophoresis. Separation gels were 12 % acrylamide/0.01 % SDS in 0.5 M Tris-HCl pH 8.8. Stacking gels were 3.5 % of acrylamide in 1.5 M Tris-HCl, pH 6.8. A conventional buffer system (Laemmli 1970) was used with a 10× running buffer are 2 M-glycine/0.1 % SDS/0.4 M Tris, pH 8.3. Electrophoresis was carried out at 100 V for 1 h 30 min in a Slab gel system (BioRad). Gels were stained with 0.1 % Coomassie brilliant blue.

Protein identification

An aliquot from fraction 9 related to the purified PeCL from the gel-filtration was sampled and the protein was precipitated using a conventional Trichloroacetic acid (TCA)/acetone procedure. Acrylamide gel bands corresponding to the two bands from the SDS-PAGE were also sampled to be trypsin digested. Protein pellet was resuspended in 20 µl of 50 mM NH₄HCO₃, pH 8.0. Cysteines were reduced with 1 µl of a 200 mM dithiothreitol (DTT) solution in 100 mM NH₄HCO₃ for 10 min at 50 °C followed by alkylation for 45 min with 0.8 µl of a 50 mM iodoacetamide solution in 100 mM NH₄HCO₃ at room temperature in the dark. Alkylation was halted by

neutralizing the remaining iodoacetamide through the addition of 4 μl of a 200 mM DTT in 100 mM NH_4HCO_3 at room temperature for 45 min. Digestion was then performed overnight with 0.1 μg of trypsin in water. The resulting peptides were dried in a vacuum centrifuge and resuspended in 30 μl of 0.1 % formic acid in water. Peptide separation by reversed-phase liquid chromatography was performed on an Ultimate LC system (LC Packings) complete with Famos autosampler and Switchos II microcolumn switching device for sample clean-up and preconcentration. A 30 μl sample was loaded at a flow rate of 200 nL min^{-1} on a micro-precolumn cartridge (300 $\mu\text{m i.d.} \times 5 \text{ mm}$, packed with 5 μm C18 100 \AA PepMap). After 5 min, the precolumn was connected with the separating nano-column (75 $\mu\text{m i.d.} \times 15 \text{ cm}$, packed with C18 PepMap100, 3 μm , 100 \AA) and the gradient started. Elution gradient varied from 0 % to 30 % buffer B over 30 min, buffer A is 0.1 % formic acid in acetonitrile/water 2:98 (v v^{-1}) and buffer B is 0.1 % formic acid in acetonitrile/water 20:80 (v v^{-1}). The outlet of the LC system was directly connected to the nano electrospray source of an Esquire HCT ion trap mass spectrometer (Bruker Daltonics, Germany). Mass data acquisition was performed in the mass range of 50–1700 m z^{-1} using the Standard-Enhanced mode (8100 m z^{-1} s^{-1}). For each mass scan, a data-dependant scheme picked the four most intense double- or triple-charged ions to be selectively isolated and fragmented in the trap and the resulting fragments were mass analysed using the Ultra Scan mode (50–3000 m z^{-1} at 26 000 m z^{-1} s^{-1}). Raw data were analysed and formatted (Data Analysis software, Bruker) for subsequent protein identification against the NCBI nonredundant protein database through the MS/MS ions search algorithm on the Mascot search engine (www.matrixscience.com). The mass tolerance of precursor and sequence ions were set at 0.5 and 0.3 Da, respectively, and carbamidomethylation of cysteines and methionine oxidation were set as fixed and variable modifications, respectively.

Aphid rearing and in vitro toxicological testing

Myzus persicae aphids used in this study were reared for many years on broad beans (*Vicia faba* L.) at $20 \pm 2^\circ\text{C}$, 16 h photoperiod before being used for artificial diet bioassays. The composition of the diet and the chamber methodology used in all experiments was the A5 diet (Febvay *et al.* 1988) with a sucrose content adjusted to 15 %. Diet was filtered through a 20 μm membrane and used immediately or aliquots are stored at -20°C until used. Diet sachets (two layers of parafilm enclosing 150 $\mu\text{g ml}^{-1}$ of diet) were changed every 2 d. Twenty neonate aphids (24 h old) in five replicates (total of 100 insects) were deposited on artificial diets supplemented with the purified PeCL lectin at 0.1 % (w v^{-1}). Aphid mortality rates were determined for up to 5 d using a range from 0 to 2 mg ml^{-1} of PeCL or ConA (Sigma, Bornem, Belgium) and compared to lectin-free diet. All diet transfers and manipulations were performed under sterile conditions.

Statistical analysis

Results from the toxicological assays were analyzed by Minitab Software (version 13.1) using analysis of variance and Tukey mean test.

Results

Lectin purification

A chromatogram relating to *Penicillium chrysogenum* lectin (PeCL) shows a peak of haemagglutination activity presented in Fig 1. A summary of PeCL yield and efficiency is shown in Table 1.

Fractions 8–11 showed high haemagglutination activity. The specific haemagglutinating activity of the crude extract was obtained at 86.4 HAU mg^{-1} , and after purification by gel-filtration, the specific activity was increased up to 1062.0 HAU mg^{-1} for fraction 9. A more than 12-fold purification ratio was obtained.

Haemagglutination inhibition assays

To characterise the carbohydrate-binding specificity of PeCL, different sugars were used to assess the haemagglutination activity of the purified fungal lectin (Table 2). An inhibition of the haemagglutination activity was observed from 39.1 μg of added mannose. None of the other tested carbohydrates showed any inhibition of the haemagglutination at up to 312.5 μg sugar.

The active purified fractions (fraction 9 and 60 % precipitation) were subjected to SDS-PAGE for molecular mass and quality purification. Two bands, of 31 and 40 kDa respectively, were observed and related to the FPLC-Sephadex G-75 column purified PeCL (Fig 2).

Protein identification

According to the LC-MS-MS analysis, the identified peptides, namely ISAV, KTKTSS, LVEAE, LGGVG, FTPAELAHH were not found to show significant homology when compared to the available sequences from the NCBI database. Only one positive match was obtained with a mannose-binding lectin from *Zea mays* (Accession number AAZ30382) based on the VAVQDDG amino acid sequence.

Toxicity assays using purified PeCL

Toxicological assays were carried out to assess the potential effect of the PeCL purification fractions on *Myzus persicae* mortality on alternate days up to 5 d (Fig 3). Significant differences in mortality were observed between PECL and control diet fed aphids after 5 d ($F = 5.96$, $P < 0.05$) mainly, with PeCL gel-filtration fraction 9 after 1 d ($t = 3.67$ and $P < 0.05$), 3 d ($t = 5.98$ and $P < 0.001$) and after 5 d ($F = 15.75$, $P < 0.001$). No significant difference in aphid mortality related to the two controls, namely the lectin-free diet and the diet including inert fraction 35 (containing PBS). *Myzus persicae* mortality was higher when fed on artificial diet containing 0.1 % PeCL than aphid fed with 0.1 % ConA common lectin reference, after 3 and 5 d ($t = -3.25$, -3.96 , $P < 0.05$).

A range of PeCL concentrations (from 0.1 to 2 mg ml^{-1} , w v^{-1} corresponding to 0.01–0.2 % lectin concentrations) from the most active fraction (fraction 9) were incorporated into artificial diet and fed to *M. persicae*. The LC50 value was determined to be 0.4 mg ml^{-1} of purified PECL lectin. A comparison with the reference ConA lectin was performed and demonstrated the

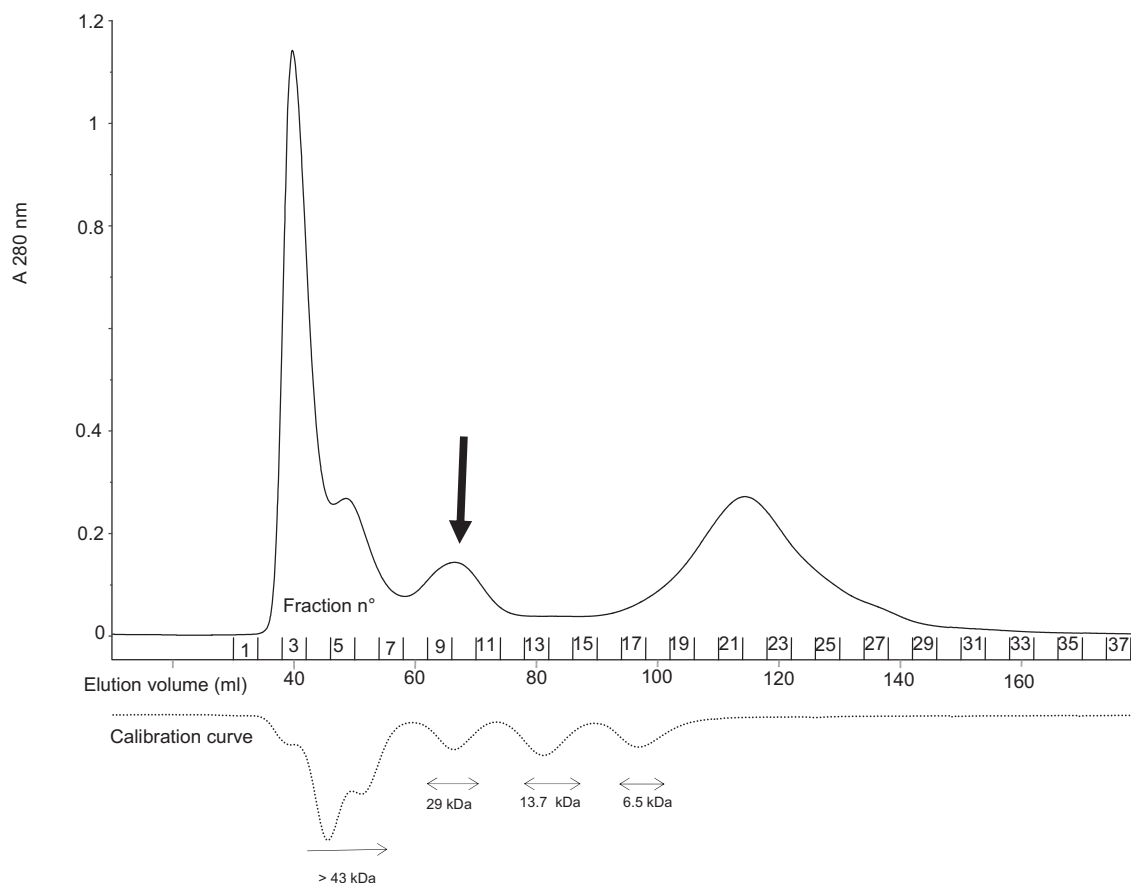


Fig 1 – Chromatogram related to the lectin purification from *Penicillium chrysogenum* by gel-filtration. The arrow indicates the active lectin fraction.

relatively high toxic effect of PECL on *M. persicae* compared to ConA (Fig 4).

Significant differences in *M. persicae* mortality were observed when fed with PeCL and ConA in artificial diets after 1 d at 0.1 % ($F = 6.02$, $P < 0.05$), after 3 and 5 d from 0.5 % ($t = 6.54$, 5.50 and $P < 0.05$). High significant differences were observed in aphid mortality rates at highest dose, 0.2 % corresponding to 2 mg ml^{-1} , when PECL and ConA treatments were compared after 5 d ($F = 16.07$, $P < 0.001$). Therefore, the effect on *M. persicae* of both lectins differed, PeCL showed higher toxic efficiency than the ConA reference lectin.

Discussion

Fungi contain various potential insecticidal proteins, including ribosome inactivating proteins, proteases, nucleases and lectins (Ng 2004). Until now, a range of mushroom lectins including, *Xerocomus chrysenteron* lectin (XCL), *Agaricus bisporus* lectin (ABL), and *Arthrotrichy oligospora* lectin (AOL) have been isolated and all of which induce reversible antiproliferative effects on different cell types (Wang et al. 1998; Oda et al. 2003; Ng 2004). Among fungi systematic, Ascomycetes are found in a broad

Table 1 – Purified fractions of lectin from *Penicillium chrysogenum* by gel-filtration.

Step	Volume (ml)	Total protein ^a (mg)	Total HAU ^b	Specific activity HAU ^b mg ⁻¹	Purification fold	Recovery activity%
Crude extract	40	23.2	3200	86.4	1.0	100
Fraction 8	4	0.9784	320	327.2	3.8	10
Fraction 9	4	0.7792	640	821.0	9.5	20
Fraction 10	4	0.6024	640	1062.0	12.3	20
Fraction 11	4	0.5408	80	147.9	1.7	3

^a Protein concentration as determined according to the method of Bradford (1976).

^b Haemagglutination activity unit.

Table 2 – Effect of various carbohydrates on haemagglutination activity from *Penicillium chrysogenum* lectin.

Sugar	Sugar quantity (μg) for one HAU ^a					
	312.5	156.3	78.1	39.1	19.5	9.8
D-Mannose	–	–	–	–	+	+
D-Galactose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
N-Acetyl-D-galactosamine (GalNAc)	+	+	+	+	+	+
N-Acetyl-D-glucosamine (GlcNAc)	+	+	+	+	+	+

–: No haemagglutination activity (inhibition).
 +: Haemagglutination activity (no inhibition).
 a Haemagglutination activity unit.

range of environments including soil, fresh- and salt-waters, or organic wastes playing a main role in ecosystems. Among the many Ascomycetes, some *Penicillium* species are grown for diverse industrial productions such as cheese, gluconic acid or several penicillin type antibiotics (particularly *Penicillium chrysogenum* for the latter). Due to the large scale production of these fungi and the availability of related industrial residues, we investigated the potential interesting proteins from *Penicillium* fungi such as lectins that could potentially be categorized as new pesticidal proteins. In this study, a lectin from the Ascomycete fungus, *P. chrysogenum* was rapidly and effectively purified. Indeed, most of lectin purification corresponded to use of combined procedures including ammonium sulphate fractionation, gel-filtration, ion exchange and affinity chromatography methods. Here, the gel-filtration step was very efficient step which was already revealed to be useful to purify mannose-glucose specific lectin such as the *Pisum sativum* lectin

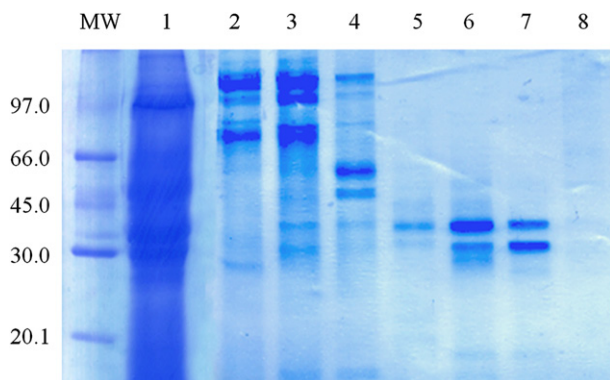


Fig 2 – SDS-PAGE of *Penicillium chrysogenum* lectin purification fractions. Lane 1: crude extract of *P. chrysogenum*, lane 2: fraction number 3 obtained during the purification, lane 3: fraction 4, lane 4: fraction 6; lane 5: fraction 8; lane 6: fraction 9; lane 7: fraction 10, and lane 8: fraction 11. Amersham molecular weight scale (MW) was added (Phosphorylase b, 97 000 Da; Albumin, 66 000 Da; Ovalbumin, 45 000 Da; Carbonic anhydrase, 30 000 Da; Trypsin inhibitor 20 100 Da; α -Lactalbumin, 14 000 Da).

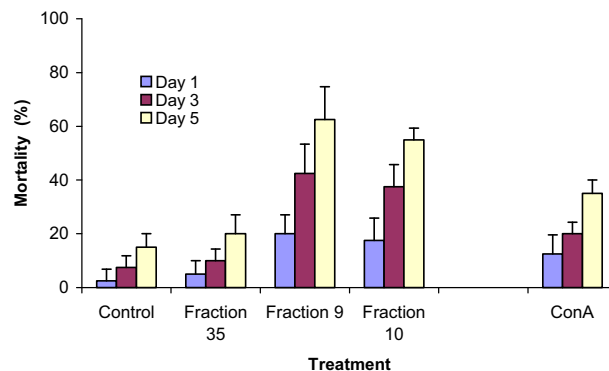


Fig 3 – Mortality of *Myzus persicae* when fed with fractions 9 and 10 from PECL purification procedure and ConA at 0.1% (w v^{-1}) concentrations. Controls include a lectin-free artificial diet and an inert fraction 35 containing PBS.

from industrial pea protein extraction residues (Cuertaro-Diaz, unpubl.). The aim of the proposed purification procedure was to obtain a highly purified, active lectin fraction using a simple, rapid and low cost technique.

With a more than 12-fold purification factor related to the most concentrated fraction, the yield obtained for *P. chrysogenum* lectin (PeCL) was in the same range that other purified fungal lectins after combined chromatography procedures and the recovery yield of PeCL was relatively higher (2–25%) than those previously reported for purified fungal lectins (Liu *et al.* 2004; Jung *et al.* 2007). Mushroom lectins previously purified were mainly proteins from Basidiomycetes such as *Agaricus*,

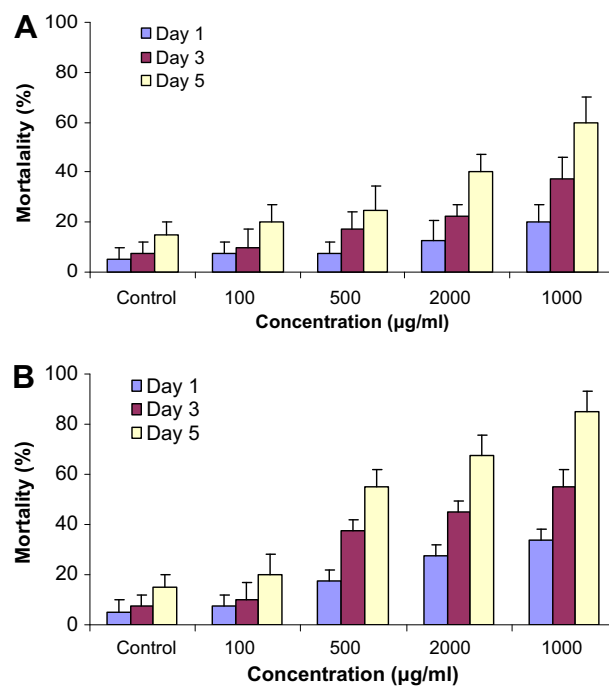


Fig 4 – Mortality of *Myzus persicae* when fed with increasing concentrations (w v^{-1}) of (A) ConA or (B) PECL where the control represents a lectin-free diet.

Pleurotus, and *Xerocomus* (Kawagishi et al. 1988; Trigueros et al. 2003; Wang & Ng 2003; Li et al. 2008). In comparison relatively few lectins have been reported from Ascomycetes, namely from *Aleuria aurantia*, *Melastiza chateri*, *Ciborinia camelliae*, *Sclerotinia sclerotiorum*, *Xylaria hypoxylon*, and *Cordyceps mimitaris* (Jung et al. 2007). No direct homology was found with available sequences of nucleic acid from *P. chrysogenum* in NCBI. Mushroom lectins have a diverse range of molecular mass and subunit number, some are dimers or tetramers with subunit molecular mass from 12 to 41 kDa, other are monomers with the same range of molecular mass (Ng & Lam 2002; Otta et al. 2002). Here two bands were observed on SDS-PAGE with 31 and 40 kDa weight. These bands were cut and the polypeptides were trypsin digested before being analysed by mass spectrometry. Both bands were found to correspond to lectin. PeCL seems to be derived from differential processing such as glycosylation of the lectin such as for ConA in plant.

Sugar-binding specificity of purified PeCL was tested according to several carbohydrate inhibition tests and revealed that our lectin was mannose specific. A sequence homology was identified with a mannose lectin from *Zea mays* according to the mass spectrometry analysis. Mannose-binding lectins are considered as biologically important proteins because mannose is widely distributed in microorganisms and animals including insects (Wong et al. 2008). Based on our previous works where fungal lectins were identified with potential insecticidal properties (Francis et al. 2003; Karimi et al. 2006; Karimi et al. 2007) the potential toxic effect of PeCL was investigated on aphids. PeCL lectin was shown to be highly toxic to *Myzus persicae* when compared to the lectin ConA which is known to have glucose- and mannose-binding properties. Similar entomotoxic property was observed by Trigueros et al. (2003) using another mushroom with *Xerocomus chrysenteron* lectin (XCL) when *Drosophila melanogaster* fly and *Acyrtosiphon pisum* aphid were fed with XCL in artificial diets. After a comparison of XCL and ConA towards two aphid species, XCL was more aphicidal than ConA (Karimi et al. 2006; Karimi et al. 2007). According to the toxicological results from this study, both XCL and PeCL were found to be similarly entomotoxic and could be considered as potential insecticidal proteins to control aphids. Indeed, significant aphid mortality was observed from 0.05 % PeCL concentration in artificial diet.

Among the insect pest orders, some Hemiptera cause serious damage to many crop plants by directly extracting the nutrients from the plants but also by acting as virus vectors (Barton et al. 1987; Peferoen et al. 1990). According to the direct toxic effect of lectins on insect biological parameters (Gatehouse et al. 1984; Down et al. 2003; Sauvion et al. 2004) but also to the potential competitive effect of lectins towards viral particles in virus transmission by aphids, fungal lectins such as PeCL represent a very promising protein to control aphid pest damages in crops.

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