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Food safety assessment of an antifungal protein from *Moringa oleifera* seeds in an agricultural biotechnology perspective

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ABSTRACT

Mo-CBP$_3$ is an antifungal protein produced by *Moringa oleifera* which has been investigated as potential candidate for developing transgenic crops. Before the use of novel proteins, food safety tests must be conducted. This work represents an early food safety assessment of *Mo*-CBP$_3$, using the two-tiered approach proposed by ILSI. The history of safe use, mode of action and results for amino acid sequence homology using the full-length and short contiguous amino acids sequences indicate low risk associated to this protein. *Mo*-CBP$_3$ isoforms presented a reasonable number of alignments (>35% identity) with allergens in a window of 80 amino acids. This protein was resistant to pepsin degradation up to 2 h, but it was susceptible to digestion using pancreatin. Many positive attributes were presented for *Mo*-CBP$_3$. However, this protein showed high sequence homology with allergens and resistance to pepsin digestion that indicates that further hypothesis-based testing on its potential allergenicity must be done. Additionally, animal toxicity evaluations (e.g. acute and repeated dose oral exposure assays) must be performed to meet the mandatory requirements of several regulatory agencies. Finally, the approach adopted here exemplified the importance of performing an early risk assessment of candidate proteins for use in plant transformation programs.

1. Introduction

Fungi are responsible for several diseases that attack plants causing damages in various important crops, decreasing their productivity (Mandal et al., 2013; Labandeira and Prevec, 2014). Furthermore, the chemical control used as strategy in combating fungal diseases is associated with toxic and hazardous effects on the environment and nontarget organisms (Meng et al., 2010; Marei et al., 2012; Yadav et al., 2013). Modern strategies to confront this problem include conventional plant breeding, which is conceptually supported by crossing of plants with desired characteristics, and genetic engineering, which aims to obtain transgenic plants expressing defined characteristics (Qaim, 2010; Zhang et al., 2010; Wang et al., 2014).

Significant efforts have been directed towards the identification of antifungal proteins that can be used for producing crops resistant to pathogens. Despite the importance of this issue, no commercial transgenic plant is available with genes that encode proteins to confer resistance against phytopathogenic fungi. On the other hand, several studies have already described the efficacy of antifungal proteins when processed on different host plants, either in laboratory or greenhouse conditions (Lacerda et al., 2014).

Moringa oleifera Lamarck is a native plant from northwest India, with distribution in various parts of the world, mainly in the tropics. *M. oleifera* is known as a multipurpose tree since it has been widely used as food and feed, as well as in the traditional medicine and industry (Ramachandran et al., 1980; Anwar et al., 2007; Ben Salem and Makkar, 2009; Kumar et al., 2010). From the seeds of this plant species, a chitin-binding protein, *Mo*-CBP$_3$ (*M. oleifera* – Chitin-Binding Protein), has been purified. *Mo*-CBP$_3$ possesses a broad spectrum of activity against important phytopathogenic fungi, such as *Fusarium oxysporum*, *Fusarium solani*,

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Rhizoctonia solani, *Colletotrichum gloesporioides* and *Colletotrichum musae* (Gifoni et al., 2012). As to its structural attributes, *Mo*-CBP$_3$ is a glycoprotein with apparent molecular mass of 18.0 kDa, presenting multiple heterodimeric isoforms composed of two different polypeptide chains linked by disulfide bonds. It is a highly stable protein, maintaining its secondary structure and antifungal activity at extremes of temperature and pH (Batista et al., 2014). In this context, *Mo*-CBP$_3$ protein may represent a promising tool for use in the development of transgenic crops resistant to phytopathogenic fungi. Indeed, the Embrapa (Brazilian Agricultural Research Corporation) group has considered the inclusion of this protein in its plant transformation programs. However, this company in a partnership with our team has agreed to perform early food safety assessments of candidate proteins before their introduction into crops. This could avoid the use of inappropriate protein for costly activities involved in the production of transgenic plants as well as may help to guide modifications in the protein structure in order to free it of its potential risks. Current strategies for food safety assessment of candidate proteins are essentially based on the guidelines of FAO and WHO compiled in the second edition of the Codex Alimentarius document (Codex Alimentarius, 2009).

The International Life Sciences Institute (ILSI) proposed a well-accepted alternative guideline (Delaney et al., 2008). It is a two-tiered evaluation, based on weight of evidence, to assess the safety of novel proteins. The steps consist of potential hazard identification of the protein (Tier I) and hazard characterization (Tier II), performed when the results of the first stage are not sufficient to define safety. Tier I includes an assessment of the biological function or mode of action and intended application of the protein, history of safe use, comparison of the amino acid sequence of the protein to other proteins, biochemical and physicochemical properties, as well as the expression level and dietary intake of the recombinant proteins. Tier II comprises acute and repeated dose toxicological studies and/or hypothesis-based testing. In fact, the ILSI guideline is a more flexible approach because it takes into account all data obtained in a holistic way. The predictive value of each piece of evidence should be well understood in order to give some data more 'weight' than others during the assessment, adding greater confidence in overall evaluation (Delaney et al., 2008).

Although the two-tiered approach, based on weight of evidence, proposed by ILSI has been initially planned for proteins in a stage of the development of the transgenic crop where the protein expression levels can be estimated, we also believe on their suitability for the safety assessment of new proteins whose use in plant transformation is still being analyzed. Thus, the present study aimed to perform an early food safety assessment of the antifungal protein *Mo*-CBP$_3$ in an agricultural biotechnology perspective, following this approach. This study allowed us to gather information about the *Mo*-CBP$_3$ risks that were crucial to formulate decisions on the immediate use of this protein for plant transformation.

2. Material and methods

2.1. M. oleifera seeds

To obtain the protein of interest, *Mo*-CBP$_3$, *M. oleifera* pods were collected from trees in the Pici Campus at the Federal University of Ceará (Fortaleza, Brazil). A voucher specimen was deposited in the Prisco Bezerra Herbarium (Fortaleza, Brazil), at this same Institution, under number EAC34591. The seeds were then separated from the pods and kept in plastic containers at room temperature until the moment of analysis.

2.2. Mo-CBP$_3$ preparation

Mo-CBP$_3$ was purified from crude extract of *M. oleifera* mature seeds as well described previously by Gifoni et al. (2012). Briefly, the crude extract was extensively dialyzed against Milli-Q grade water and $(NH_4)_2SO_4$ added to the soluble material, denoted as the albumins, to yield 90% saturation. Albumin fraction was dissolved in and dialyzed against the extracting buffer and applied to a chitin column that had been equilibrated with the same buffer. After elution with the starting buffer of the unbound proteins from the column, the chitin-bound proteins, named P_{NAG} and P_{AC}, were eluted with 0.1 M *N*-acetyl-D-glucosamine (NAG) that was prepared in the extracting buffer and with 0.05 M acetic acid (pH 5.0), respectively. The P_{NAG} sample was dialyzed against 0.1 M acetic acid and distilled water, lyophilized and applied to a cation-exchange matrix (Resource S™) that had been previously equilibrated with 0.05 M sodium acetate buffer, pH 5.2. Four major adsorbed protein peaks (*Mo*-CBP$_2$, *Mo*-CBP$_3$, *Mo*-CBP$_4$, and *Mo*-CBP$_5$) were recovered after being selectively desorbed by stepwise elution with 0.4, 0.5, 0.6, and 0.7 M NaCl, respectively, included in the equilibrium buffer.

The production of *Mo*-CBP$_3$ was monitored through quantification of soluble proteins by Bradford method (Bradford, 1976), using a curve constructed with bovine serum albumin as standard. The purification of the protein was monitored by 17.5% SDS-PAGE (Laemmli, 1970). Briefly, *Mo*-CBP$_3$ (1 mg/mL) was mixed to 2× sample loading buffer [62.5 mM Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue] (1:1, v/v) and incubated at 100 °C for 5 min. Then, 2.5 μg of protein/well were run at 20 mA/gel for 1 h. Protein bands were visualized by coomassie brilliant blue R-250 staining.

From the SDS-PAGE profile of *Mo*-CBP$_3$, the percentage of relative purity of the protein was calculated using the Image Master 2D platinum (v.7.0, GE Healthcare) software. This methodology has been adopted by our team, and it is also widely used by chemical companies to show the purity of commercialized proteins.

Immediately after the production of the *Mo*-CBP$_3$ and up to 72 h before its use in the *in vitro* and *in vivo* tests, batches of the protein were checked to verify the maintenance of their bioactivity against fungi according to the methodology described by Gifoni et al. (2012).

2.3. History of safe use of M. oleifera species

A literature review on the history of safe use (HOSU) of *M. oleifera* species, source of the *Mo*-CBP$_3$ protein, was performed according to the principles described by Constable et al. (2007). For that search, the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) was accessed, using the following combination of keywords: (a) "*Moringa oleifera*" and "food safety"; (b) "*Moringa oleifera*" and "risk assessment"; (c) "*Moringa oleifera*" and "toxicity"; (d) "*Moringa oleifera*" and "nutrition"; (e) "*Moringa oleifera*" and "review"; and (f) "*Moringa oleifera*".

2.4. Amino acid sequence similarity

From cDNA sequences (GenBank accession numbers KF616830 until KF616833) coding for *Mo*-CBP$_3$ were deduced the amino acid sequences of four isoforms of this protein referred to as *Mo*-CBP$_3$-1, *Mo*-CBP$_3$-2, *Mo*-CBP$_3$-3, and *Mo*-CBP$_3$-4 (Freire et al., 2015). These sequences were used in a FASTA format without the signal peptide and compared with all protein sequences deposited in seven large reference public databases: NR, Refseq_Protein, SwissProt, PDB, Env_nr (http://www.ncbi.nlm.nih.gov/), UniProt SwissProt (http://www.uniprot.org/) and Uniprot-trEMBL (http://www.expasy.org/). The algorithm used was BLASTP 2.2.29+ and the scoring matrix was BLOSUM62. No keyword was used to limit the search. Specific