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A new TRAF-like protein from *B. oleracea* ssp. *botrytis* with lectin activity and its effect on macrophages

Christiane E.M. Duarte[a,1], Monise V. Abranches[b,1], Patrick F. Silva[a], Sérgio O. de Paula[a], Silvia A. Cardoso[c], Leandro L. Oliveira[a,*]

[a] Departamento de Biologia Geral, Universidade Federal de Viçosa, 36570-900 Viçosa, MG, Brazil
[b] Departamento de Nutrição e Saúde, Universidade Federal de Viçosa, 38810-000 Rio Paranaíba, MG, Brazil
[c] Departamento de Medicina e Enfermagem, Universidade Federal de Viçosa, 36570-900 Viçosa, MG, Brazil

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ABSTRACT

Lectins are involved in a wide range of biological mechanisms, like immunomodulatory agent able to activate the innate immunity. In this study, we purified and characterized a new lectin from cauliflower (*Brassica oleracea* ssp. *botrytis* – BOL) by three sequential chromatographic steps and confirmed the purity by SDS-PAGE. Additionally, we evaluated the role of the lectin in innate immunity by a phagocytosis assay, production of H_2O_2 and NO. BOL was characterized like a non-glycosylated protein that showed a molecular mass of ~34 kDa in SDS-PAGE. Its N-terminal sequence (ETRAFREERPSSKIVTIAG) did not reveal any similarity to the other lectins; nevertheless, it showed 100% homology to a putative TRAF-*like* protein from *Brassica rapa* and *Brassica napus*. This is a first report of the TRAF-protein with lectinic activity. The BOL retained its complete hemagglutination activity from 4 °C up to 60 °C, with stability being more apparent between pH 7.0 and 8.0. Moreover, the lectin was able to stimulate phagocytosis and induce the production of H_2O_2 and NO. Therefore, BOL can be explored as an immunomodulatory agent by being able to activate the innate immunity and favor antigen removal.

1. Introduction

Brassicaceae is one of the major groups of the plant kingdom, composed of 348 genera and more than 3700 species, distributed worldwide [1]. Brassica oleracea is a very morphologically diverse species, including the common heading cabbage (*B. oleracea* ssp. *capitata* L.), cauliflower (*B. oleracea* ssp. *botrytis* L.), broccoli (*B. oleracea* ssp. *italica* L.), kale and collards (*B. oleracea* ssp. *acephala*), kohlrabi (*B. oleracea* ssp. *gongylodes* L.), Chinese kale (*B. oleracea* ssp. *alboglabra*), and Brussels sprouts (*B. oleracea* ssp. *gemmifera* DC) [2]. Brassica species form an important human food crop plant with great economic value as vegetables and as sources of edible and industrial oil, animal fodder, and green manure [3].

Plants are a rich source of lectins predominantly isolated from seeds, which comprise 10% of the total protein content in a mature seed [4]. They are also present in the vegetative tissues such as leaves, fruits, roots, tubers, rhizomes, bulbs, bark, stems, phloem sap and even nectar [5]. Lectins are a group of carbohydrate-binding proteins found in viruses, bacteria and eukaryotes, which are involved in various biological processes, such as cell–cell interaction, folding of glycoproteins, host defense, self/non-self-recognition and intracellular routing [6]. Although lectins possess several biological properties in common, they represent a diversified protein group with respect to size, composition and structure.

The use of lectins for biomedical applications has grown because of research studies that indicate their antitumor properties [7] and antimicrobial activities [8] beyond the potential use of these proteins as diagnostic markers [9]. It has been shown that lectins exert an immunostimulating action such as Concanavalin A, functioning as a mitogenic agent, enabling the study of the interaction of lectin with the lymphocyte cells *in vitro* [10]. In innate immunity, soluble lectins are able to direct the antigen elimination and assist in the phagocytic action by the macrophages and dendritic cells. These proteins enhance the immune system by opsonization, culminating in the activation of the adaptive immune response, like the mannose-binding lectin present in humans [11].

In this study, we report the first isolation and characterization of a lectin from cauliflower and evaluate its biological effects on macrophage activation. Additionally, this is the first report of a lectin with only MATH-domains.

* Corresponding author.
E-mail address: leandro.licursi@ufv.br (L.L. Oliveira).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Biological material

The cauliflowers (*Brassica oleracea* ssp. *botrytis*) were purchased from different suppliers in Viçosa, Brazil. The Federal University of Viçosa provided goat, horse and ox erythrocytes and BALB/c mice. The adult male BALB/c mice (20–25 g) were maintained in a photoperiod (12 h light:12 h dark) controlled ambient environment (25 °C), with free access to water and food. This study was performed in strict accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and Brazilian Society of Animal Science Laboratory. The Ethics Committee on Animal Research of the Federal University of Viçosa approved the protocol (Permit Number: 21/2012). Human blood group A, B and O erythrocytes were collected from healthy donors at the Health Center of Federal University of Viçosain in accordance with the Committee on the Ethics of Humans of the Federal University of Viçosa (Permit Number:108/2012/CEPH/wmt).

2.2. Soluble protein extraction procedures

The cauliflowers were ground and homogenized with a vegetable crusher, in phosphate-buffered saline (PBS) (pH 7.4) in the ratio of 1:1 (w/v), and set aside at 4 °C for 8 h. The supernatant was filtered through a 0.45 μm membrane (Schleicher & Schull, German) to obtain the crude extract.

2.3. Hemagglutination activity assay

A serial two-fold dilution of the lectin (50 μL) was mixed with 25 μL of a 2% of erythrocyte suspension in microtiter U-plates. The hemagglutination titer is defined as the reciprocal of the highest dilution exhibiting hemagglutination. Specific activity is defined as the number of hemagglutination units per mg protein [12]. Hemagglutination activity was evaluated using goat, horse, ox and human A, B, and O erythrocytes.

2.4. Protein purification

The crude extracts were loaded on a HiTrap Blue HP column (0.7 cm x 2.5 cm, GE Healthcare), which had been equilibrated prior with 50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 1.0 mL/min. The bound proteins were eluted with 1 M NaCl in 50 mM Tris-HCl buffer (pH 7.4). After dialysis, the sample was subjected to ion exchange chromatography on a HiTrap Capto S column (0.7 cm x 2.5 cm, GE Healthcare) equilibrated with 50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 1.0 mL/min. The bound proteins with the hemagglutination activity were eluted with 100 mM NaCl in the 50 mM Tris-HCl buffer (pH 7.4). In the final "polishing" step, we used a Protein-Pak column (7.8 mm x 300 mm, Waters) equilibrated with 0.9% (w/v) NaCl at a flow rate of 0.7 mL/min. The absorbance in all the chromatographic steps was monitored at 280 nm.

2.5. SDS-PAGE

SDS-PAGE was performed in the presence or absence of 2-Mercaptoethanol using a 12% resolving gel and 5% stacking gel [13]. The gel was stained with 2% (w/v) Coomassie Brilliant Blue R-250. The Protein Marker 6.5–200 kDa (SERVA, Germany) was used as the standard molecular mass marker.

2.6. Protein concentration

The protein concentration was determined using the BCA *Protein Assay* kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions, using bovine serum albumin (BSA) as the standard.

2.7. Determination of protein glycosylation

In order to determine if BOL is a glycoprotein, a bioinformatics analysis was undertaken using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/) for the presence of predicted *N*-glycosylation sites and *O*-glycosylation sites [14], respectively.

2.8. N-terminal sequencing

To determine N-terminal amino acid sequence, purified protein was separated by 12% SDS-PAGE and Electroblotted at 100 mA for 1 h on to ProBlott membranes (Applied Biosystems, USA) then stained with 0.1% Coomassie for 30 s, destained with 50% methanol, washed with distilled water and dried overnight. The desired fragments were excised and sequenced. Automatic Edman degradation analyses were performed on the protein sequencer model PPSQ-33A (Shimadzu, Japan).

2.9. Mass spectrometry analysis and protein sequencing by tandem mass spectrometry

Briefly, the BOL band was cleaned of the SDS-PAGE gel, destained with 50% acetonitrile/25 mM ammonium bicarbonate, in-gel reduced with DTT 65 mM for 30 min at 56 °C and alkylated with iodoacetamide 200 mM for 30 min at room temperature. It was then dried with acetonitrile followed by SpeedVac™. Samples were digested using 50 μL of 2.5 μg/mL trypsin (Sigma) in 10% acetonitrile/40 mM ammonium bicarbonate pH 8 at 37 °C overnight. Peptides were extracted with 50% acetonitrile/5% acid formic, dried in SpeedVac™ and redissolved in 8 μL 0.1% formic acid. The samples were desalted using Zip Tip C18 (Sigma). The sample matrix used was Universal MALDI-Matrix (Sigma). Mass spectra were acquired in the reflector ion mode in the *m/z* range of 640–3240 using an Ultraflex III MALDI-TOF/TOF mass spectrometer controlled by flexAnalysis software v. 2.0 (Bruker Daltonics). The instrument was equipped with a smartbeam laser (Bruker Daltonik), and the acquisition laser power was optimized using the PS calibration mixture before collection of the sample data. The peptide masses were sought against the NCBI database employing Mascot (in-house MASCOT-server) for protein identification.

2.10. Inhibition of hemagglutination

The hemagglutination inhibition tests used various 400 mM carbohydrate solutions (D-glucose, D-galactose, D-arabinose, D-xylose, *N*-acetyl glucosamine, D-fructose, D-mannose, D-ribose, melibiose, maltose, D-lactose, D-cellobiose, D-trehalose, saccharose and D-raffinose) and glycoproteins at a concentration of 0.5 mg/mL (asialofetuin, fetuin and casein) were performed in a manner analogous to the hemagglutination test. A serial two-fold dilution of each sugar sample was prepared in PBS. All the dilutions were mixed with an equal volume (25 μL) of the lectin solution with one hemagglutination unit. The mixture was allowed to stand for 30 min at room temperature and then mixed with 25 μL of a 2% goat erythrocyte suspension. The minimum concentration of the sugar which completely inhibited one hemagglutination unit of lectin was calculated [12].

2.11. Glycoproteins proteolysis

Fetuin and asialofetuin (1 mg/mL) were digested with 50 μg/mL proteinase-K (Promega, USA) at a 1:1 (w/w) enzyme is to substrate ratio in 50 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$ at 45 °C overnight. The complete digestion was confirmed by 12% SDS-PAGE. Then, the inhibition of the lectin-induced hemagglutination was tested using digested and non-digested glycoproteins.

2.12. Effects of temperature, pH and divalent cations on lectin activity

Aliquots of lectin were incubated at different temperatures (4 °C to 100 °C) for 30 min and cooled in ice. The hemagglutination activity of the aliquots was tested. The pH stability of the lectin was measured by dialyzing the lectin aliquots against the following buffers for 6 h at 4 °C: 100 mM glycine buffer (pH 2.0 and 3.0), 20 mM acetate buffer (pH 4.0 and 5.0), 100 mM phosphate buffer (pH 6.0 and 7.0), and 100 mM glycine–NaOH buffer (pH 10.0, 11.0 and 12.0). The pH of the lectin solution was adjusted to 7.0 by the addition of 0.1 N HCl or 0.1 N NaOH before the hemagglutination activity was determined. To determine the metal ion dependence, the protein was dialyzed against 100 mM Tris-HCl, 10 mM EDTA at pH 7.4 for 12 h. Following this period, the lectin was dialyzed once again, but this time against 100 mM Tris-HCl at pH 7.4, followed by the hemagglutination assay. Additionally, the dialyzed protein fractions were dialyzed against 50 mM $CaCl_2$, 50 mM $MgCl_2$, 50 mM $MnCl_2$ or 50 mM $ZnCl_2$, followed by the hemagglutination assay.

2.13. Phagocytic activity of the peritoneal macrophages

Macrophages from the peritoneal cavity of the BALB/c mice were suspended with RPMI culture medium (Gibco, USA), supplemented with 10% fetal bovine serum, 100 units penicillin/mL and 100 mg streptomycin/mL. A 200 μL aliquot of this cell suspension (10^5 cells/100 μL/well) was seeded into a well of a 6-well plate and covered with a coverslip. This was followed by incubation for 2 h at 37 °C in a humidified atmosphere of 5% CO_2. Different concentrations of the lectin in 200 μL of complete RPMI medium were then added to the wells followed by incubation for 30 min. After that, a *Pichia pastoris* (5×10^5 cells/well) suspension was added and the plates were incubated for 2 h. The supernatant was removed and 400 μL of 10% formaldehyde in PBS was added. The coverslips were stained with HEMA 3 Panoptic dye (Renylab, Brazil) and analyzed with a light field optical microscope (Olympus, Japan).

2.14. NO production by peritoneal macrophage assay

Macrophage from the BALB/c mice peritoneal cavity were washed and resuspended in the RPMI culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/mL and 100 mg streptomycin/mL. The cells were seeded in a 96-well culture plate (2×10^5 cells/well) and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 2 h. The cells were stimulated with different concentrations of lectin or (2.5 mg/mL) Zymosan (positive control), followed by incubation for 48 h. The supernatant was collected and the amount of nitric oxide in the culture medium was determined by the colorimetric method [15].

2.15. H_2O_2 production by the peritoneal macrophage assay

Macrophages from the BALB/c mice peritoneal cavity were washed with PBS and suspended in phenol red buffer (140 mM NaCl, 10 mM potassium phosphate, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/mL peroxidase type II, pH 7.0). The cell aliquots (100 μL) were seeded in a 96-well culture plate and incubated with

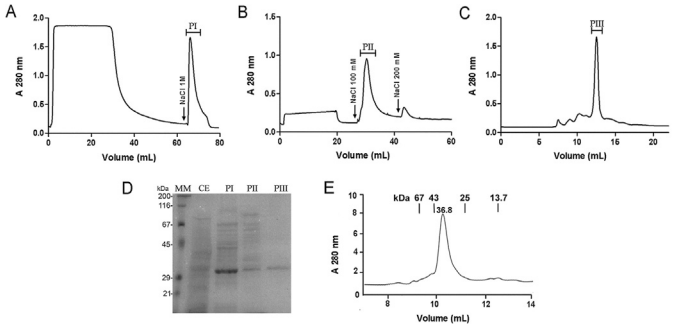

Fig. 1. Purification of cauliflower lectin. (A) Affinity chromatography of crude extract of cauliflower on a HiTrap Blue HP column. The peak labeled PI exhibited HA. (B) Ion exchange chromatography of fraction PI on a HiTrap Capto S column. The peak labeled PII exhibited HA. (C) Molecular size exclusion chromatography of fraction PII on a Protein-Pak column. The peak labeled PIII exhibited HA. All the elutions were monitored at 280 nm. (D) The SDS-PAGE of the fractions obtained in the chromatography steps. MM: molecular weight marker, CE: crude extract, PI-fraction obtained by affinity chromatography, PII-fraction obtained by ion exchange chromatography, PIII-fraction obtained by gel filtration. (E) Estimation of molecular weight by gel filtration, using BSA, ovalbumin, chymotrypsinogen A and ribonuclease A as calibration standard.

different concentrations of lectin or (2.5 mg/mL) Zymosan for 1 h at 37 °C in a humidified atmosphere with 5% CO_2. The reaction was stopped by the addition of 10 μL/well of 1 M NaOH. The H_2O_2 present in the medium was determined by the colorimetric method [16].

2.16. Statistical analyses

The statistical significance was analyzed using the analysis of variance (ANOVA), followed by the Dunnett test, using the GraphPad Prism® version 5.0 software. Differences with $p < 0.05$ were considered statistically significant. All experiments were performed in triplicate.

3. Results

3.1. Protein purification

Purification of the cauliflower lectin involved the initial extraction in PBS (pH 7.4) and three-step chromatography including affinity chromatography on the HiTrap Blue HP column, ion-exchange chromatography on the Mono S column, and gel filtration on the Protein-Pak column. Fractionation of the crude extract using HiTrap Blue HP revealed the presence of a slightly smaller adsorbed fraction, designated as PI (Fig. 1A). This fraction, with hemagglutination activity, was subsequently applied on the Mono S column, by means of which a fraction designated as PII (Fig. 1B) was obtained. The adsorbed fraction with hemagglutination activity was resolved into a large peak (PIII) by gel filtration on the Protein-Pak column (Fig. 1C). The purified lectin, represented by PIII, appeared as a single band with an apparent molecular mass of 34 kDa on SDS-PAGE (Fig. 1D) and a 36.8 kDa on gel filtration (Fig. 1E), these results reinforce the observation that lectin is a monomeric protein. A gradually enriched lectin was purified and then designated as *Brassica oleracea* ssp. *botrytis* lectin (BOL). An almost 139-fold purification and a recovery of 12% were achieved through the purification process (Table 1).

3.2. Properties of purified lectin

The physical and biochemical properties of the lectin were investigated. BOL migrated as a single band on the SDS–PAGE under reducing and non reducing conditions (Fig. 2A). Taken together

Table 1
Specific hemagglutination activities and chromatographic fraction yields obtained at different stages of lectin purification.

Purification steps	Total Protein (mg)	Total Activity (HA)	Specific Activity (HA/mg)	Purification fold	Recovery (%)
Crude extract	10642.50	180000	17	1.0	100
HiTrap Blue HP	395.28	43200	109	6.4	24
HiTrap Capto S	80.28	28800	359	21.1	16
Protein-Pak	8.91	21120	2370	139.4	12

HA – Hemagglutination Activity Unit, corresponds to the minimum quantity of protein capable of inducing agglutination; HA/mg corresponds to the amount of hemagglutination units per milligram of protein.

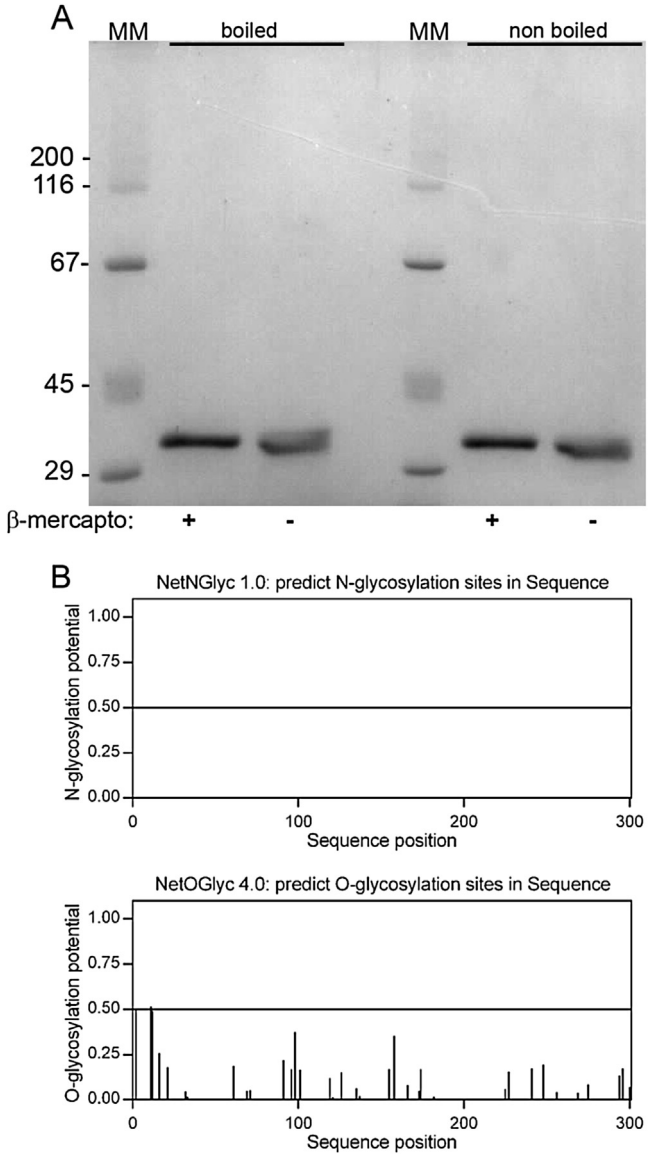

Fig. 2. Properties of the purified BOL. BOL is a monomeric lectin (A) SDS–PAGE under the reducing and non-reducing conditions. Lanes 1 and 4, molecular weight markers, Lanes 2 and 3: samples boiled. Lane 2, reducing condition and Lane 3 non-reducing condition. Lanes 5 and 6: not boiled samples. Lane 5 reducing condition, Lane 6 non-reducing condition. BOL is a non-glycosylated protein (B) *In silico* prediction of possible sites of glycosylation using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 4.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/).

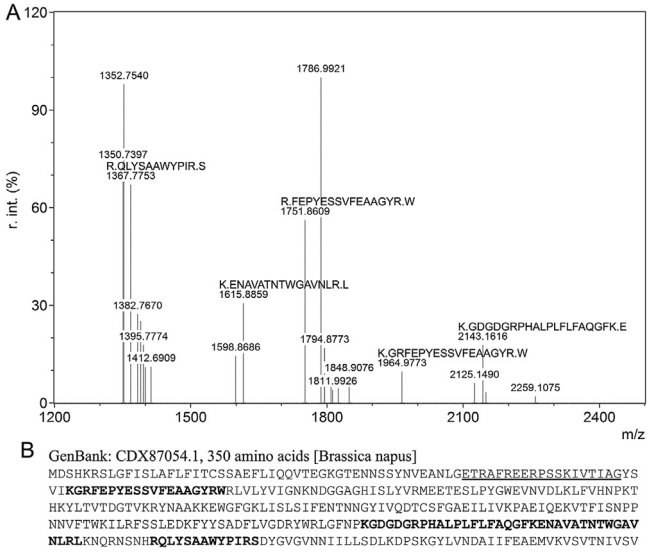

Fig. 3. Mass spectrometric analysis of BOL. A) Mass fingerprint obtained from tryptic digested BOL was analyzed by MALDI-TOF/TOF mass spectrometer scanning from 640 to 3240 amu in the positive ion mode for detection of protonated peptides. Each tryptic peptide was subjected to LIFT dissociation to produce a fragment ion pattern and the amino acid sequence was deduced. The tryptic peptides are listed above the ion pattern. B) Amino acid sequence of putative protein of Brassica napus (CDX87054.1). N-terminal (residues 1–19) determined by Edman degradation (underline) and tryptic peptides by mass spectrometry (bold).

with gel filtration results we can conclude that BOL is a monomeric protein. The *in silico* prediction of possible sites of glycosylation show that BOL has no N-glycosylation sites and a minimal probability of being *O*-glycosylated, so BOL is a non-glycosylated protein (Fig. 2B). The N-terminal amino acid sequence of BOL was obtained by the automated Edman degradation. The first 19 amino acid residues were determined (ETRAFREERPSSKIVTIAG) which showed significant homology by the BLAST to predict, and uncharacterized proteins from *Brassica rapa* (XP_009111696.1) and *Brassica napus* (CDY19775.1 and CDX87054.1) with 100% identity with a putative TRAF-*like* protein.

The purified protein was digested by trypsin and the resultant peptides were analyzed by mass spectrometry (MALDI-TOF/TOF). Fig. 3 shows the monoisotopic masses of the five peptides identified, which were used to identify the homologous proteins in the NCBI database through the MASCOT server. Matching the same set of peptides aligned with the homologous sequences of the 39.5 kDa putative TRAF-*like* protein of *Brassica napus* (CDX87054.1) and *Brassica rapa* (XP_009111696.1) was achieved with the 309 MASCOT score.

3.3. Carbohydrate specificity of the purified lectin

The blood specificity of BOL was determined by use of erythrocytes from different species (goat, horse, ox) and humans from the ABO system. The lectin showed more selective for horse and goat erythrocytes than others (Fig. 4A). The hemagglutination activity of the purified cauliflower lectin was not observed to be inhibited by any of the simple sugars tested at 400 mM; however, it was inhibited by the glycoproteins, asialofetuin > fetuin > ferritin > casein, but not for ovalbumin (Table 2). To determine if the interaction of the BOL-glycoproteins was mediated by a carbohydrate-protein

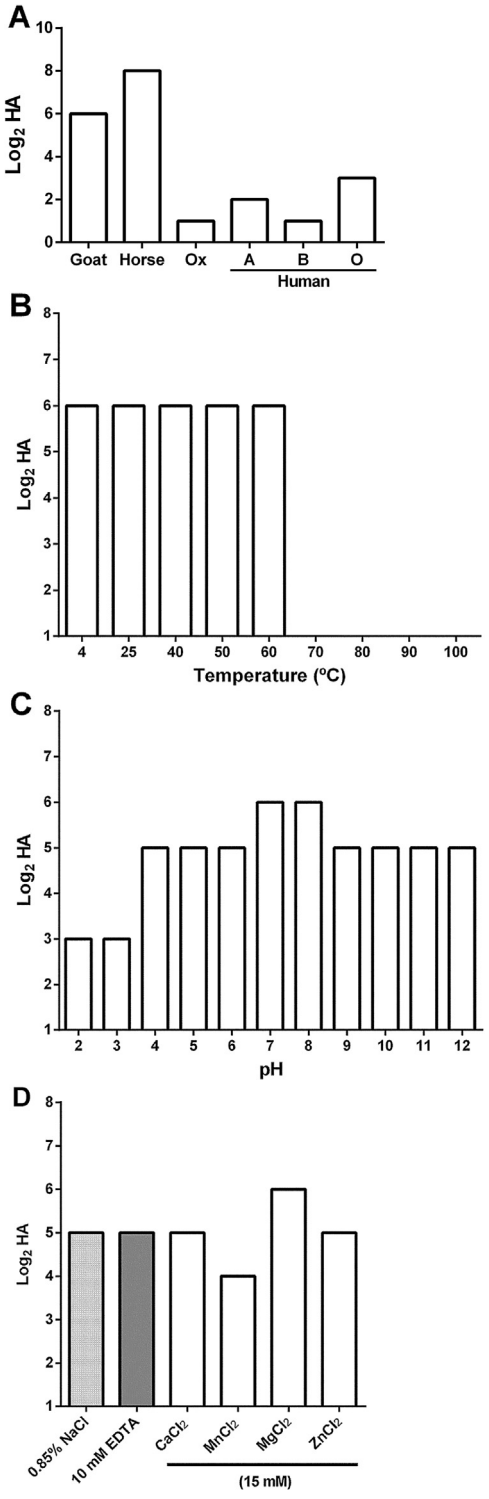

Fig. 4. Physicochemical characterization of BOL. (A) Specificity of agglutination activity, red blood cells of goat, horse, ox and human, A,B and O groups were tested. (B) Thermal stability of BOL. The lectin was incubated at an elevated temperature (4–100 °C). (C) pH stability of BOL. The lectin was incubated with buffers ranging from pH 2.0 to 12.0. (D) Influence of the divalent cations on BOL. After treatment with a chelating agent, the lectin was incubated with the indicated various divalent cations. The bars represented the HA of BOL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effects of the various carbohydrates and glycoproteins on the hemagglutination induced by the *B. oleracea* lectin.

Inhibitor	mM
Simple sugars[a]	ND
Asialofetuin	0.12
Asialofetuin + proteinase K[b]	0.24
Fetuin	0.23
Fetuin + proteinase K[b]	0.46
Casein	1.30
Ferritin	0.57
Ovalbumin	ND

ND-inhibition non-detected.

[a] Lactose, galactose, arabinose, melibiose, xylose, cellobiose, N-acetyl glucosamine, fructose, glucose, maltose, mannose, saccharose, ribose, trehalose, raffinose were non-inhibitory at 400 mM concentration.

[b] Glycoprotein (1 mg/mL) was digested with 50 μg/mL proteinase-K (overnight, 45 °C).

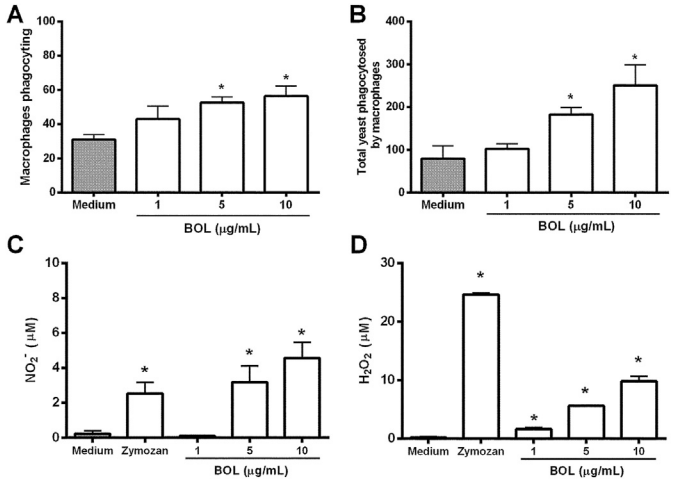

Fig. 5. Macrophage Activation. Peritoneal macrophages of BALB/c mice were treated with the lectin obtained from cauliflower. (A) The number of macrophages that exhibited phagocytosis in each of the 200 cells analyzed. (B) Phagocytic index in each of the 200 macrophages analyzed. (C) Nitric oxide production by the macrophages. (D) Hydrogen peroxide production by the macrophages. Assays were performed in triplicate; the results represent the average ± SD of three independent experiment; $^{*}p < 0.05$ compared with the control group.

or protein–protein interaction, fetuin and asialofetuin complete proteolysis were performed (Data not show) and the hemagglutination activity of the BOL was noted to continue to be inhibited by oligosaccharides (Table 2).

3.4. Effect of temperature and pH

The thermal stability of BOL was determined in the temperature range between 4 and 100 °C. The results indicated that BOL was stable between 4 and 60 °C. The lectin was totally inactivated when incubated at 70 °C for 30 min (Fig. 4B). The pH sensitivity profile of the lectin is shown in Fig. 4C, in which the stability was more apparent between pH 7.0 and 8.0. The hemagglutination activity of the native lectin was not affected either by the sequential dialysis (with EDTA followed by Tris-HCl) or by the addition of Ca^{2+} and Zn^{2+} to the dialyzed lectin. The lectinic activity was slightly inhibited in the presence of Mn^{2+} and increased after the addition of Mg^{2+} (Fig. 4D).

3.5. Cauliflower lectin activates macrophages and promotes phagocytosis

In order to verify whether BOL was capable of acting as an immunostimulator, we evaluated the induction of macrophage activation promoted by the BOL. The effect of cauliflower lectin on the phagocytic activity of the peritoneal macrophage in engulfing yeast cells is shown in Figs. 5A and 5B. The phagocytosis of the yeast cells is increased by two-fold ($p < 0.05$) when compared with the control. The BOL induced a significant increase in the production of inflammatory mediators compared with the untreated cells. The results of the nitrite and H2O2 production are shown in Figs. 5C and 5D. Taken together we can see that the macrophages were activated by lectin, phagocytizes more, better and with greater capacity microbicide.

4. Discussion

The isolation, purification, characterization and biological applications of plant lectins have been the focal point of several studies over the last few years [17–19]. The protocol used in the purification can be distinguished into three sequential chromatography steps, enabling the recovery of 12% of the total hemagglutination activity present in the crude extract, with 139-fold purification. BOL was found to be a non-glycosylated monomer, with a molecular mass estimated at 34 kDa protein by SDS-PAGE and 36.8 kDa by gel filtration.

BOL does not exhibit sequence similarity with the other earlier reported lectins, including the lectin isolated from broccolini [20]. According to the search results from N-terminal homology and MALDI-TOF/TOF the lectin showed 100% identity with a putative TRAF-*like* protein of *Brassica rapa* and *Brassica napus*. The TRAF family, a tumor necrosis factor of receptor-associated factors, was first identified as a group of mammalian adaptor proteins. TRAF proteins physically and functionally connect the cell surface receptors to the signaling pathways involved in the regulation of diverse cellular responses, which include activation, differentiation and survival [21]. This type of protein also was seen in plants (*Arabidopsis*, *Medicago*, *Oryza*, and *Sorghum*), lower eukaryota (*Trypanosoma*, *Dictyostelium*, *Theileria* and *Plasmodium*), and lower metazoa (*Caenorhabditis elegans*) [22]. However, until now, TRAF-like proteins with lectin activity have not been described. BOL was related as a hypothetical protein that possess only two MATH-domains without any other protein domain, the function of these kind of proteins not yet know. BOL can be the first TRAF-like protein capable of recognizing carbohydrates, and we hypothesize that the MATH domain can be a new carbohydrate-recognition domain (CRD).

The *B. oleracea* lectin was not inhibited by the mono-, di- or tri-saccharides, but complex carbohydrate structures inhibited its activity. Asialofetuin and fetuin were found to be strong inhibitors of the lectin from *B. oleracea* suggesting that the BOL binds the complex N-linked oligosaccharides. To demonstrate that the inhibition of hemagglutination activity of BOL was caused by oligosaccharide moieties, the asialofetuin and fetuin were completely hydrolyzed by proteases and the lectin-inhibition was maintained. Wright et al. [23], also observed the inhibition of the hemagglutination activity by the fetuin and asialofetuin, which in turn inhibits the action of the lectin obtained from *Scilla campanulata*. Although plant lectins have specificity toward monosaccharides, they show high specificity to the more complex glycans that are found in animals and humans but absent from plants [5]. Recent high performance analytical techniques (like glycan microarray analysis) demonstrated that plant lectins have a preferential binding to oligosaccharides and glycans rather than to monosaccharides. Even lectins classic as GNA, which was originally considered a mannose-specific lectin, interact only weakly with mannose but exhibit a strong afinity to high-mannose N-glycans [24]. This property may be related to the fact that lectins are capable of recognizing the glycoconjugates present on the microorganism surface or in digestive tracts of insects and herbivorous animals and are possibly part of plant defense pathways [5,25].

The lectin retained its whole hemagglutination activity from 4 °C up to 60 °C. The BOL activity was also maintained in wide pH variation, with stability being more evident between pH 7.0 and 8.0, while 50% activity remained at pH 4–6 and 9–12. A similar case is observed with the lectins from other plant species, for example *Glycine max* [26] and *Phaseolus coccineus* [27]. Lectins are mostly the defense proteins [6] which are known for their stability under various physicochemical conditions [28]. On the other hand, some lectins have been reported whose activities decrease above pH 9.0 [29] or below pH 5.0 [30]. The stability shown by BOL increases the applications of this protein.

Purified lectin does not need bivalent cations to reveal the hemagglutination action. Lectin activity remained unaltered even after metal ion chelation with EDTA or in the presence of Ca^{+2} and Zn^{+2} ions; however, it was affected by the Mg^{2+} and Mn^{2+} ions. The hemagglutination activity of *Inocybe umbrinella* lectin was also depressed by Mn^{2+} [31] while the activity of Con A was potentiated by the Ca^{2+} and Mn^{2+} ions [32]. Divalent cations although it does not required for the formation of heterodimers, may increase the stability of the complex formed by decreasing the dissociation rate [33]. In addition, antimicrobial activity of peptides can also be increased in the presence of divalent ions which excess may induce conformational changes in the peptide [34]. Therefore, the influence of the divalent cations in the binding of BOL to the carbohydrates could be explained in the light of the appropriate conformational recognition.

The immunomodulatory effect triggered by the cauliflower lectin was evidenced by its capacity to stimulate the phagocytosis and production of the inflammatory mediators by the peritoneal macrophages. Wong and Ng [15] reported that the banana lectin increased the NO production by the macrophages, in a dose-dependent manner. Similar results were observed with onion lectin, which induced a significant increase in the production of NO, the pro-inflammatory cytokines and phagocytic activity of the yeast cells by the activated macrophages [19]. In our study, the cauliflower lectin activated the macrophages by inducing the NO and H_2O_2 production. Unitt and Hornigold (2011)[35] reported that some plant lectins exhibit specific patterns of stimulation of human Toll-*like* receptors, suggesting that the innate immune system can detect and respond to certain lectins. Working in this direction, Mariano et al. [36] presented a plausible mechanism of macrophage stimulation: ArtinM, a D-mannose-binding lectin, interacted with Toll-*like* receptor 2 and its heterodimers in a carbohydrate recognition-dependent manner, which culminated in a larger secretion of cytokines, due to the action of the NFκB nuclear transcriptional factor. Furthermore, several plant lectins exhibit immunomodulatory activities that are initiated by their interaction with the glycan moieties present on the surfaces of the immune cells. Such interactions may trigger signal transduction to produce certain cytokines and induce efficient immune responses against tumors or microbial infections [37].

5. Conclusion

This is the first report of the isolation of a lectin from Brassica oleracea ssp. botrytis (BOL). In this study we purified, characterized and evaluated the stimulatory effects of BOL, which demonstrated be able activate macrophage improve their clearance capacity.

It is also described for the first time a TRAF-like protein with lectin activity, supporting the concept that the lectins are indeed multifunctional and diverse group. The new lectin isolated from cauliflower can favoring the removal of foreign agents, which is a potentially exploitable activity

Author contributions

L.L.O. and S.A.C. designed and coordinated the study. L.L.O., C.E.M.D. and M.V.A. wrote the paper. M.V.A., C.E.M.D. and P.F.S. performed and analyzed the experiments. S.O.P. and S.A.C. provided technical assistance and contributed to the preparation of the figures and tables. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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