An Acad Bras Cienc (2021) 93(3): e20191402 DOI 10.1590/0001-3765202120191402
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Beta-1,3-glucanase inhibitors in Brazilian brown seaweed

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Abstract: Beta-1,3-glucanases are enzymes that hydrolyze beta-1,3-glucans, and they are essential for the metabolism of seaweed, plants and fungi. These enzymes also participate in the digestion of herbivore and fungivore animals. Because of the importance of these enzymes in insects, beta-1,3-glucanase inhibitors may be used for the development of new control strategies against agricultural pests and disease vectors. Beta-1,3-glucanase inhibitors have been described in the brown seaweed *Laminaria cichorioides*, but were never recorded in Brazilian seaweed species. We evaluated the presence of beta-1,3-glucanase inhibitors in samples of *Padina gymnospora*, *Dictyota* sp., *Colpomenia sinuosa*, and *Lobophora* sp., collected in Arraial d'Ajuda (Bahia). Ethanolic or buffer extracts were used in inhibition tests against the beta-1,3-glucanase of *Trichoderma* sp. Extracts in buffer showed no inhibition, but ethanolic extracts from all species showed different extents of inhibition. Samples from *Dictyota* sp. and *P. gymnospora* showed inhibitions above 75% (absolute ethanol) or 50% (ethanol 50%). In summary, extraction with absolute ethanol resulted in better inhibitions, and *P. gymnospora* showed the higher inhibitions. Brazilian seaweed may be good sources of beta-1,3-glucanase inhibitors for biochemical and physiological studies of these enzymes. Besides that, these molecules show potential for the development of new biotechnological tools for insect control.

Key words: β-1,3-glucanase, inhibitor, Seaweed, *Padina gymnospora*, *Trichoderma*.

INTRODUCTION

Beta-1,3-glucans are polysaccharides, consisting mainly by glucose subunits linked by beta-glycosidic bonds between carbons 1 and 3 of adjacent residues (beta-1,3 linkages). They may also contain beta-1,4 glycosidic bonds, in mixed linear β-1,3-1,4-glucans as lichenan or cereal β-glucans, or β-1,6 ramifications, as in the fungal and yeast β-1,3-1,6-glucans. There is a great variety of these polysaccharides in nature, occurring in seaweed, plants, yeast and fungi, mainly as a component of the cell wall (Burtseva et al. 2003, Genta et al. 2003, 2009, Rop et al. 2009).

In brown seaweed, β-1,3-1,6 glucans, named as Laminarin, act as reserve polysaccharides, with a role that is similar to amylose in terrestrial plants. These algae may synthesize inhibitors against the digestive β-1,3-glucanases of marine animals, avoiding predation in a strategy similar to that used by terrestrial plants that produce amylase inhibitors against herbivory (Ermakova et al. 2001, Yermakovaet al. 2002). A β-1,3-glucanase inhibitor with high specificity and activity against β-1,3-glucanases from marine mollusks has been isolated from the brown seaweed *Laminaria cichoriodes* (Ermakova et al. 2001, Yermakova et al. 2002).

β-1,3-glucanases are frequently found in the gut of detritivore and herbivore insects, being likely that they perform important functions in the digestive process of these organisms (Moraes et al. 2012, Terra & Ferreira 2005). These enzymes were already described in insects of several orders as Dictyoptera (Genta et al. 2003, Lucena et al. 2011), Coleoptera (Genta et al. 2009) Orthoptera (Genta et al. 2007), Lepidoptera (Bragatto et al. 2010) and Diptera (Moraes et al. 2012, 2014, Souza et al. 2016, 2019). Importantly, these enzymes seem to be essential for the development of agricultural pests as the mealworm *Tenebrio molitor* and the fall armyworm *Spodoptera frugiperda*, and disease vectors as the phlebotominae sandfly *Lutzomyia longipalpis* and the yellow fever mosquito *Aedes aegypti*. Insect gut β-1,3-glucanases may also perform a role in the innate immune system, by recognition of fungal pathogens and activation of the prophenoloxidase cascade (Pauchet et al. 2009). The inhibition of β-1,3-glucanases in insects is highly deleterious, impairing the development of larvae and increasing mortality by opportunistic pathogens (Souza et al. 2019, Bulmer et al. 2009).

Plants commonly use the inhibition of digestive enzymes for protection against predators. Enzyme inhibitors with action against insects have been used for crop protection (Clemente et al. 2019). In this context, the discovery of new targets and inhibitors might contribute for the development of new strategies for insect control. In this respect, we screened Brazilian seaweeds as a potential new source for β-1,3-glucanase inhibitors, that may be further explored in biotechnological applications. We were able to describe β-1,3-glucanase inhibition using ethanolic extracts of four different seaweed species, with the best results obtained with *Padina gymnospora*. Therefore, Brazilian seaweed may be in the future a promising source of enzyme inhibitors for blocking the digestion of detritivore and herbivore insects of economic importance.

MATERIALS AND METHODS

Reagents and samples

The brown seaweed were collected in March of 2012 in the coast of Arraial d'Ajuda, in the municipality of Porto Seguro, Bahia State, Brazil. Four different species were collected: *Colpomenia sinuosa*, *Dictyota* sp., *Lobophora* sp. and *Padina gymnospora*. Samples were stored in 15 mL Falcon tubes with silica gel at -20ºC.

For preparation of the extracts, we used Absolute Ethanol (Merck), and Phosphate Buffer Saline (PBS, Sigma). In the enzyme assays the substrate used was the β-1,3-glucan Laminarin from *Laminaria digitata* (Sigma, Cat. No. L9634) and the enzyme was the β-1,3-glucanase from *Trichoderma* sp. (Sigma Cat. No. L5272). For reducing sugars detection we used Dinitrosalicilic Acid (DNS, Sigma Cat. No. D0550) or Bicinchoninic Acid (BCA, Sigma Cat. No. D8284).

Samples were incubated in the oven at 37ºC for 24h for desiccation and the contents weighed. The dried tissues were divided in three equal parts and stored in 1.5 mL polypropilene tubes at -20ºC. These subsamples were extracted separatedly with absolute ethanol (Et), ethanol 50% (v/v) (Et50%) or PBS 20 mM pH 7. The conditions for the preparation of each extract are specified in Table I .

Each subsample was grounded with ceramic mortar and pestle in liquid nitrogen, being suspended in 2 mL of the solvents above. Samples in absolute ethanol or ethanol 50% (v/v) were kept at room temperature for 3 days, and to avoid bacterial growth samples in PBS were incubated at 4ºC for the same amount of time. After this, the samples were centrifuged at

Table I. **Information about the samples prepared from four different species of brown seaweed, and results from enzyme assays and inhibition in the presence of the extracts. Seaweed *Lobophora* sp., *Colpomenia sinuosa, Padina gymnospora* and *Dictyota* sp. were used for extraction in different solvents (PBS – phosphate buffer saline pH 7, 20 mM; Et – absolute ethanol; Et 50% - Ethanol 50% (v/v)). Weights are the mass of tissue used for extraction in each sample, g/mL is the final concentration of tissue in solvent during extraction, % inhibition is the mean decrease of activity when *Trichoderma* β-1,3-glucanase is incubated with the seaweed extract. Abbreviations: ns: not shown. Activity in controls, activity with extracts and activity inhibited are the absolute values of β-1,3-glucanase measured in each set of experimental conditions.**

Sample ID	Species	Dry weight (g)	Solvent	[g/ mL]	% Inhibition	% Inhibition, mean	Mean activity in controls (μU/ mL)	Mean activity with extracts (μU/mL)	Mean Activity Inhibited, μU
1	***Colpomenia sinuosa***	0.09	Et	0.05	0	0	10 ± 1	25 ± 3	0
2		0.07		0.04	0				
4		0.09		0.04	0				
1		0.05	Et 50%	0.02	0	29	16 ± 5	11 ± 2	4
2		0.06		0.03	22				
4		0.08		0.04	64				
4		0.14	PBS	0.07	0	0	ns	ns	ns
1	***Dictyota* sp.**	0.09	Et	0.05	100	42	16 ± 3	11 ± 6	0-5
2		0.07		0.04	6				
3		0.06		0.03	19				
1		0.10	Et 50%	0.05	0	51	12 ± 0.4	7 ± 4	5
2		0.09		0.05	100				
3		0.07		0.03	52				
			PBS		0	ns	ns	ns	ns
4	***Lobophora* sp.**	0.13	Et	0.07	100	71	20 ± 6	6 ± 3	14
8		0.12		0.06	75				
9		0.12		0.06	37				
4		0.13	Et 50%	0.07	17	16	13 ± 2	11 ± 2	2
8		0.07		0.04	0				
9		0.08		0.04	31				
9		0.07	PBS	0.03	0	0	ns	ns	ns
1	***Padina gymnospora***	0.19	Et	0.09	49	68	10 ± 2	3 ± 1	7
7		0.19		0.09	100				
8		0.20		0.10	54				
1		0.19	Et 50%	0.09	68	47	15 ± 4	8 ± 2	7
7		0.28		0.14	24				
8		0.26		0.13	50				
1		0.16	PBS	0.08	0	na	na	na	na

4ºC for 10 seconds at 2,000 g and their soluble fractions were collected. These fractions were kept until use in criopreservation tubes at -20ºC.

Enzyme Assays

Stock solutions of β-1,3-glucanase (0.1 mg/mL) in PBS 20 mM pH 7 and laminarin (0.5%, w/v) in deionized water (Milli Q, Millipore) were prepared and kept frozen in -20ºC.

Assays were assembled by mixing 5 µL β-1,3-glucanase, 5 µL laminarin, 5 µL sample (soluble fraction of the extracts), and 10 µL PBS 20 mM pH 7. Each assay included controls with 5 µL solvent (ethanol, ethanol 50% or PBS) instead of sample. A second set of assays was performed with 10 x dilutions of each extract in its respective solvent. Assay replicates were incubated at 30ºC for 0, 15, 30, 45 or 60 minutes. After incubation, each tube was flash frozen in an ethanol/dry ice bath and kept at -20ºC.

Reducing sugars were detected with DNS or BCA as described in Lucena et al. (2013). One unit of activity correspond to the amount of enzyme that generates 1 µmol of product per minute.

RESULTS

All data of this screeening, including detailed sample information (seaweed species, part used for the extraction, dry weight, solvent, concentration) and inhibiton results for the β-1,3-glucanase activity from *Trichoderma* sp. (control activity in the presence of solvent and % inhibition) were summarized in Table I. Interestingly, samples from all seaweed species tested showed inhibition of the β-1,3-glucanase.

In general, all assays showed linear increment of absorbance with time (data not shown), indicating that the extracts do not have any compound that inactivates the *Trichoderma* enzyme in the conditions used. Besides that, the varibility among controls was low (SEM values between 7.4-12.5% of means, see below), showing great reproducibily. However, a significant inhibition effect due to the presence of ethanol alone was observed, with 0.0032 ± 0.0004 Abs/min in controls with ethanol 50% (v/v) *versus* 0.0027 ± 0.0002 Abs/min in controls with absolute ethanol.

Preliminary results with samples prepared with *Lobophora sp*. and *P. gymnospora* in phosphate buffer saline showed no inhibition (Table I), so we decided to not include this condition for the screening of other species. Extracts prepared with ethanol 50% and absolute ethanol showed inhibition to different extents, with the sole exception of *C. sinuosa* in absolute ethanol (Table I). Inhibition with extracts in absolute ethanol ranged from 42 to 71%, with the highest inhibition observed with *Lobophora* sp. Extracts in ethanol 50% resulted in inhibitions ranging from 16 to 51%, with the highest values obtained with *P. gymnospora* (Table I). Ten times dilution of all extracts in their respective solvents resulted in loss of inhibition (data not shown).

DISCUSSION

Extracts from *C. sinuosa* in absolute ethanol have not shown inhibition. However, extracts from the same samples in ethanol 50% showed significant results, including a dose dependent response, with higher inhibition values in extract prepared with more concentrated samples. These results suggests the presence of an inhibitor with amphipathic properties.

In contrast, samples from *Dictyota* sp. showed higher inhibition values, both in ethanol and ethanol 50%, but a concentration effect was not observed. This may be related to variability in the production of inhibitors or differential

extraction efficiency in each sample. Besides that, there was no important difference in the inhibitory activity when comparing ethanol and ethanol 50%, suggesting good solubility in more hydrophobic solvents.

Extracts prepared from *Lobophora* sp. showed higher inhibition power when the samples were extracted with ethanol, when comparing to extracts in ethanol 50%. This suggests that the inhibitor molecules might have an hydrophobic nature, being less soluble in water. This is consistent with the previous report from Yermakova et al. (2002), that observed ethanolic inhibitory fractions mainly with β-1,3-1,6-glucooligosaccharides and proteins. The same was not observed in ethanol 50%.

In *P. gymnospora* the same effect was not observed, with inhibition in both ethanolic and ethanol 50%, and absence of a dose dependent response. A slightly higher inhibiton was observed in absolute ethanol.

We observed large differences when comparing the inhibition efficiencies of samples from different batches of seaweed of the same species/solvent pair. This resulted in high SEM values for some measurements. In general, absolute ethanol extractions resulted in higher inhibition values, with less variation between batches. This suggests that the seaweed β-1,3-glucanase inhibitors have hydrophobic properties, which is consistent with the fact that most interactions between β-1,3-glucanases and their ligands have hydrophobic nature (Yermakova et al. 2002).

It is possible that the differences between the inhibition power of batches from the same seaweed species are related to heterogeneities in the initial samples, that might be formed by different parts of the stalk or fruiting bodies of the plant. Besides that, the samples might correspond to seaweed exposed to very different environmental conditions as water temperature, brightness, submersion or predation by mollusks and crustacea. Ermakova et al. (2001) and Yermakova et al. (2002) described huge seasonal variations in the inhibitory capacity of extracts from the seaweed *Laminaria cichorioides*. Seasonal and taxonomical variations in the amount of laminarin and inhibitory potential were also described in different species of *Laminaria* sp. (Schiener et al. 2014, Zvyagintseva et al. 2003) which might account for the differences observed among our samples. However, the presence of β-1,3-glucans or β-1,3-linked oligosaccharides in our ethanolic extracts is unlikely, because these polysaccharides are rather insoluble in absolute ethanol. Previous reports have shown that the major sugar constituents of ethanolic extracts of brown seaweed are monosaccharides (Mian & Percival 1973), and detailed studies of their fractions have shown that inhibition of β-1,3-glucanases is not associated to the extracted sugars (Yermakova et al. 2002). Further characterization of the algae studied here is necessary to understand the parameters that influence the inhibitory activity in each species, the chemical nature of the inhibitors, and their type of inhibition on the tested β-1,3-glucanase.

The seaweed that showed the best and more consistent results for the inhibition of the β-1,3-glucanase of *T. reesei* was the brown seaweed *P. gymnospora*, followed by *Lobophora* sp. and *Dictyota* sp. All these species are promising sources for further studies and molecular characterization of the inhibitory molecules. An interesting perpective for these molecules is their use in the inhibition of insect digestive β-1,3-glucanases, which are present in almost all insect orders (Bragatto et al. 2010, Genta et al. 2003, 2007, 2009, Lucena et al. 2011). Despite the fact that the role of these enzymes have not been fully characterized, they seem to be involved in the degradation of fungal

polysaccharides, as described in the larvae of *Lutzomyia longipalpis* (Moraes et al. 2012, 2014) and *Aedes aegypti* (Souza et al. 2016, 2019). Initial experiments of inhibition with seaweed extracts against the β-1,3-glucanase from *L. longipalpis* gut extracts were performed (data not shown), but the minimal amount on enzyme obtained from this tiny insect precluded the screenings. In this respect, recombinant heterologous expression of the insect targets would be necessary. Another possibility is choosing insect models that are better sources of this enzyme, as *Tenebrio molitor* (Genta et al. 2009), *Periplaneta americana* (Genta et al. 2003), or *Aedes aegypti* (Souza et al. 2019).

CONCLUSIONS

Ethanolic extracts from the brown seaweeds *P. gymnospora* and *Lobophora* sp. showed a significant inhibitory action against the commercial β-1,3-glucanase from *T. reesei*. This might be explored for further biochemical and physiological studies aiming the inhibition of the same enzyme in insects of economical importance in Brazil, as disease vectors and agricultural pests.

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How to cite

FERREIRA TN, BARUFI JB, HORTA PA, CASTRO DP & GENTA FA. 2021. Beta-1,3-glucanase inhibitors in Brazilian brown seaweed. An Acad Bras Cienc 93: e20191402. DOI 10.1590/0001-3765202120191402.

Manuscript received on November 19, 2019; accepted for publication on March 16, 2020

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Author contributions

TNF and FAG conceived the study. TNF performed the experiments and data analysis. JBB and PAH collected and identifyied the seaweed. DPC gave important intellectual contributions. TNF and FAG wrote the initial draft and all authors revised and approved the manuscript in its final form.