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Structural characterization of the carbohydrate and protein part of arabinogalactan protein from *Basella alba* stem and antiadhesive activity of polysaccharides from *B. alba* against *Helicobacter pylori*

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Abstract

Background

Increasing drug resistance of *Helicobacter pylori* has highlighted the search for natural compounds with antiadhesive properties, interrupting the adhesion of *H. pylori* to stomach epithelia. *Basella alba*, a plant widely used in Asian traditional medicine, was investigated for its antiadhesive activity against *H. pylori*.

Methods

B. alba extract FE was prepared by aqueous extraction. Polysaccharides were isolated from FE by ethanol precipitation and arabinogalactan-protein (AGP) was isolated with Yariv reagent. Carbohydrate analyses was performed by standard methods and sequence analysis of the protein part of AGP by LC-MS. *In vitro* adhesion assay of fluorescent-labelled *H. pylori* J99 to human AGS cells was performed by flow cytometric analysis.

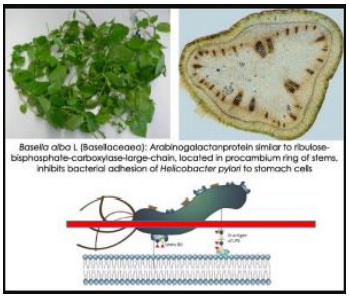
Results

Raw polysaccharides (BA1) were isolated and 9% of BA1 were identified as AGP (53.1% neutral carbohydrates L-arabinose, D-galactose, rhamnose, 5.4% galacturonic acid, 41.5% protein). After deglycosylation of AGP, the protein part (two bands at 15 and 25 kDa in tricine SDS-PAGE) was shown to contain peptides like ribulose-bisphosphate-carboxylase-large-chain. Histological localization within the stem tissue of *B. alba* revealed that AGP was mainly located at the procambium ring. Functional assays indicated that neither FE nor BA1 had significant influence on viability of AGS cells or on *H. pylori*. FE inhibited the bacterial adhesion of *H. pylori* to AGS cells in a dose dependent manner. Best anti-adhesive effect of ~67% was observed with BA1 at 2 mg/mL.

Conclusion

The data obtained from this study characterize in part the mucilage and isolated polysaccharides of *B. alba.* As the polysaccharides interact with the bacterial adhesion, a potential uses a supplemental antiadhesive entity against the recurrence of *H. pylori* after eradication therapy may be discussed.

Graphical abstract



1. Introduction

H. pylori, a Gram-negative, spiral shaped bacterium, has been classified by WHO as a group I carcinogen [1]. The first step in the development of *H. pylori's* pathogenicity is the specific recognition of the host cells, followed by a receptor-mediated adhesion process to the gastric epithelium. Therefore, *H. pylori* needs to rapidly cross the viscous mucus layer, which covers the gastric epithelial cells, to reach the epithelium surface underneath the mucus, where the pH is neutral. Once it reaches the epithelial surface, it can persist asymptomatically or can trigger cell physiology toward strong inflammation by releasing several factors, which cause host tissue damage [2]. *H. pylori* expresses several virulence factors that mediate the pathogenicity and the potential disease outcome after infection. Beside flagella, a supporting tool for mobility and work as a screwdriver during the infection, urease helps the bacterium to overcome the acidic condition of the gastric environment. For this reason, urease-inhibiting drugs are assessed to be valuable tools as anti-virulence compounds against *H. pylori* [3]. Adhesins play an additionally crucial role to maintain the specific colonization of the host cell by *H. pylori*. The adhesion to epithelial cell as well as the persistence of *H. pylori* in the stomach is mainly mediated by several outer membrane proteins (OMP) [4]. Amongst them, the most intensively studied adhesins are BabA (also known as HopS) and SabA (also known as HopP). BabA is thought to act as an important factor for initial colonization [5] since it is involved in *H. pylori* binding to MUC5AC and MUC5B present in the mucus layer. Normally, BabA interacts with fucosylated oligosaccharides present in H-1 and Lewis^{b} (Le^{b}) blood group antigens [6,7]. In addition, SabA interacts and binds to antigens such as sialyl-Lewis^{a} and sialyl-Lewis^{x}, which are generally expressed in the inflamed gastric tissue [8]. SabA further acts as an hemagglutinin, responsible for sialic acid-dependent hemagglutination [9]. Further adhesins as HopB, HopC, HopH and HopZ are also known to be associated with bacterial adhesion [[10], [11], [12], [13]]. However, their cognate receptors have not been identified in detail, yet. Interestingly, probiotic supplementation has been shown to improve the infection severity with *H. pylori*, and it is assumed that probiotic organisms strongly interact with the adherence of *H. pylori* to the gastric epithelium [14].

Treatment of *H. pylori* infection can be performed by standard antibiotic regimes, but strong recurrence rates limit the efficacy. Traditional medicine uses unspecific acting herbal materials or natural products, *e.g.,* volatile oil-containing plants [15], which can have good inhibitory values under *in vitro* conditions, but fail in many cases within clinical practice. The lifelong elimination of *H. pylori* by antibiotics faces the problem of increasing antibiotic resistance, and vaccination prophylaxis against *H. pylori* is still in development. An actual review on standard diagnosis and therapy is displayed by [14], indicating also that therapeutic intervention always depends on an individualized diagnosis and assessment of the patient. Therefore, the discovery of compounds, which specifically interact with *H. pylori* virulence factors, especially with adhesion factors, offer the use of a cytoprotective strategy against *H. pylori* and more importantly, such antiadhesive compounds might prevent recurrence after standard eradication therapy. Many antiadhesive compounds have been

found so far, including peptides [16], polyphenols [17,18], *N*-phenylpropenoyl-L-amino acid amides [19], and polysaccharides [[20], [21], [22], [23]] that interact with bacterial OMP. However, in many cases it is quite difficult to obtain enough of these natural products at a reasonable price so that drug-registered antiadhesive drugs are lacking. One of the alternative approaches is the search for natural polysaccharides that possess antiadhesive properties, interrupting the adhesion of *H. pylori* to the human stomach tissue. One example in that respect are the pectin-like rhamnogalacturonans in Okra's mucilage fresh extract from immature fruits of *Abelmoschus esculentus* [[23], [24], [25]].

B. alba L. from the plant family Basellaceae, also commonly named as Ceylon-spinach, is widely used as a traditional vegetable in Asian countries like in Vietnam, Thailand, China, and India. Due to the high amount of mucilage, *B. alba* has also been used for medical treatment for diseases such as pruritus and burn in India [26], as anticancer agent against melanoma, leukemia and oral cancer [27], to treat acne and freckle treatment in Bangladesh [28], and as a topical formulation for irritant, bruise, ringworm, and laboring in Thailand [29]. The high mucilage content of the leaves and stems of *B. alba* is obvious during preparation of aqueous extracts [30]. As in the past decade polysaccharides and glycoproteins have been recognized widely within the field of glycobiology as important signaling factors for regulation of manifold functions and pathways in cell physiology [31], the detailed investigation of *B. alba* polysaccharides seems interesting, especially within the background of the traditional medicinal use of the plant.

The structural elements of *B. alba*'s mucilage have been roughly characterized as a mixture of polysaccharides, containing arabinose, rhamnose, galactose, galacturonic acid and glucose (ratio 24:5:41:13:16) with D-galactose as the major carbohydrate. In general, high arabinose and galactose ratios might be a hint for the presence of arabinogalactans, which are known from previous studies to have strong antiadhesive properties against *H. pylori* [32]. As AGP have recently identified as selective antiadhesive compounds against *H. pylori*, interacting with BabA outer membrane proteins of the bacterium, it seems worth to investigate a potential *B. alba* AGP against *H. pylori* [32]. Therefore, the present investigation aimed to characterize a potential antiadhesive effect of *B. alba* polysaccharide-containing aqueous extract, the isolated raw polysaccharides and a fraction containing arabinogalactan protein AGP against *H. pylori* under *in vitro* conditions).

2. Materials and methods

2.1. Plant material and general methodology

If not stated otherwise all chemicals were purchased from Sigma (Deisenhofen, Germany) and VWR (Darmstadt, Germany). *B. alba* was obtained from the Botanic Garden-Vietnam National University of Agriculture, Vietnam (**Supplementary Data Fig**. **S4**). Identification was performed by HG and AH. A voucher species is deposited under code number IPBP 490 in the archives of the Institute of Pharmaceutical Biology and Phytochemistry (IPBP), University of Münster, Germany.

2.2. Preparation of fresh extract (FE) and raw polysaccharides (RPS)

The *B. alba* (BA) fresh extract (FE) was prepared according to Lengsfeld et al., [33]. Accordingly, 136.68 g fresh immature BA's stem were grounded in a mortar, followed by extraction with the 10 folds amount of Millipore® water for 6 h at 4 °C. After centrifugation (14.800 ×*g*, 30 min at 4 °C) and dialysis (cellulose tubes, Roth, MWCO 3.5 kDa) against water for 3 days at 4 °C the resulting extract was centrifuged (14.800 *g*, 30 min at 4 °C) to remove insoluble particles. The supernatant was aliquoted in 1 mL FE portions and stored at −80 °C for further use.

High molecular weight compounds from FE were precipitated in ethanol, by adding FE dropwise to the 4 × volume of ice-cold ethanol 96% (*v*/v) under continuous stirring overnight at 4 °C according to Wu et al., 1995 [34]. The resulting precipitate (RPS) was recovered by centrifugation (450 ×*g*, 1 min) and washed twice with 80% (v/v) ethanol. RPS was dissolved in water, dialyzed against Aqua Milipore® (MWCO 3.5 kDa) for 72 h at 4 °C, centrifuged at 5300 ×*g* for 10 min to remove any insoluble residue and finally lyophilized. Lyophilized RPS was named as BA1 fraction.

2.3. Precipitation of AGP from BA1 by β-D-glucosyl Yariv reagent

Arabinogalactan proteins (AGP) were isolated from BA1 by precipitation with β-D-glucosyl-Yariv reagent (1,3,5-tris-[4-β-glucopyranosyl-oxyphenylazo]-2,4,6-trihydroxybenzene) [35] as described by Sehlbach et al., [36]. Yariv reagent can effectively be used for selective precipitation of AGP; no interaction will occur with arabinogalactans without protein moiety or with glycoproteins or polysaccharides with different structural features as the typical arabinosylated 1,3-linked galactan chains, linked to the protein core [37]**.** The so obtained AGP was named BA2. Yariv reagent had been synthesized according the protocol from Yariv, Rapport and Graf (1962) [38].

2.4. Carbohydrate analysis

Standard methods of carbohydrate analysis (carbohydrate quantification, uronic acids, polysaccharide and protein hydrolysis, TLC and HPAEC-PAD for carbohydrates, GC–MS analysis of alditol acetates, D/L-configuration) were performed according to methods previously described by Hermann et al., [39] and Sehlbach et al., [36]. Carbohydrate content was analyzed by resorcinol sulphuric acid assay [40]. In this assay, polysaccharides are hydrolysed under strong acidic conditions to the respective monosaccharides. Ketoses and aldoses are subsequently hydrolyzed to furfural and 5-hydroxymethylfurfural, respectively, which subsequently react with resorcinol by forming a reddish colored xanthene, that can be detected by UV/VIS spectroscopy at λ = 450 nm. For investigation of BA2 three concentrations (100, 250, 500 µg/mL) were investigated within three independent assays.

The total amount of uronic acids was determined by colorimetric assay as described by Blumenkrantz and Asboe-Hansen [41], with modification to work in 96 well microplates. In principle, the polysaccharides are acid-hydrolyzed in the presence of sodium tetraborate. After addition of *o*-hydroxydiphenyl a colored chromogen is formed. Calibration was performed using galacturonic acid reference standard. BA2 samples were measured at two different concentration levels (250 and 500 µg/mL, six technical replicates).

D−/L-configuration was determined *via* capillary electrophoresis according to the protocol described by Noe & Freissmuth [42].

2.5. Semiquantitative determination of AGP staining with Yariv reagent

The assay is based on single radial gel diffusion method [37,38]. To prepare two plates, 500 mg agarose, 439 mg sodium chloride, 10 mg sodium azide, and 0.5 mg β-D-glucosyl-Yariv reagent were dissolved in 50 mL water and heated to 70 °C until the solid components had completely dissolved (2 min). The hot suspension was poured into two lids of a 48-well-plate. After the gel cooled down, holes of equal diameters of 1.5 mm were punched into each well using a glass Pasteur pipette. The wells were filled up with 2 µL of the test compounds and incubated overnight at 4 °C in a tightly closed plastic bag in the dark. Defined red halos formed around the wells, which is the indicator for the presence of AGP. Quantification is performed by measuring the diameter of the outside edge of the red ring formed in well subtracting for the diameter of the hole (1.5 mm). A calibration curve was

established in each plate using a linear concentration of Gum arabic fraction (c = 0.25, 0.5, 1.25, 2.5, 5 mg/mL).

2.6. Histological investigations: AGP staining in *B. alba* stem

Fresh stems of *B. alba* were cut with a custom-made sledge microtome into sections of 75–100 µm, followed by staining with aqueous β-D-glucosyl-Yariv reagent solution (0.5 mg/mL in sodium chloride 0.15 M) for 18 h. The stained sections were washed 5 × with miliQ water to remove unbound Yariv. Microscopic images were taken employing a fluorite objective (PlFl 4×/0.14) on a Leica Orthoplan microscope equipped with a Canon 5D II DSLR camera. Altogether sixty-five pictures were taken to compose the whole picture of the *B. alba* stem.

2.7. Deglycosylation of BA2 by trifluoromethanesulphonic acid (TFMS)

This method was based on Edge et al., 2003 [43] with some modification. Accordingly, 1 mg of BA2, prior dried *in vacuo* over phosphorous pentoxide, was dissolved in 1.8 mL of ice-cold anisole/TFMS acid (1:2, *v*/v) in a glass vial. The vial was flushed with a stream of nitrogen and stirred for 24 h at 0 °C. After that, 8 mL of ice-cold diethyl ether/hexane (9: 1, v/v) were added and kept at −20 °C for 12 h. Precipitated deglycosylated protein was collected by centrifugation (17,000 ×*g*, 1 min). The pellet was washed twice with ice-cold ethanol 96% with centrifugation after each time. The remaining ethanol was removed by a stream of nitrogen.

2.8. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of AGP

Protein moiety in AGP was analyzed by Tris-SDS-Page according to Laemmli (1970) [44] using "Mini-PROTEAN TGX Stain-Free TM Precast Gels" consisting of 10% polyacrylamide (Bio-Rad, U.S.A). Samples were loaded at a protein concentration 1 mg/mL. Electrophoresis started with 70 V in the stacking gel and was increased up to 100 V to finish the separation (after 1 h). Protein marker: Precision Plus Protein Multi Color, Bio-Rad, U.S.A.).

2.9. Tryptic in-gel digestion of gel bands

Deglycosylation of BA2 was performed by trifluoromethanesulfonic acid (TFMS) as described by Edge et al., 2003 [43]. Under this acidic condition, only *O*-glycosyl bonds are cleaved, while the peptide bonds are protected by the addition of anisole. Accordingly, the linkages between sugars and the core protein *via* serine, threonine and hydroxyproline are hydrolyzed**.** Gel bands were washed, destained and tryptically digested as described before [45]. Proteins were reduced, alkylated, and tryptically digested in the gel. Peptides were extracted, dried, and redissolved in 10 µL 0.1% formic acid containing 5% acetonitrile. High-definition MS was performed using a Synapt G2 Si ion mobility mass spectrometer coupled to M-Class UPLC (Waters Corp.) with a 30-min gradient (solvent system 100% water *vs.* 100% acetonitrile, both containing 0.1% formic acid; trap column V/M Symmetry C18 100 Å 5 µm, 180 µm × 20 mm; reversed phase column HSS T3 1.8 µm, 75 µm × 200 mm; and 4.5 µl injection volume). Data were analyzed with PLGS software (Waters Corp.) using the UniProt database for protein identification.

2.10. Agar diffusion assay

200 µL of *H. pylori* suspension (OD_{550} 0.2) were spread on a Tryptic Soy Agar and five sterile disks (BD Sensi-Disk, Ø 6 mm) were circularly distributed. Twenty µL of sample solutions were added on each disk. On each plate, amoxicillin solution (2.5 µg/mL) and PBS were used as positive and negative control, respectively. The plate was turned upside down and incubated under microaerophilic

conditions (5% O_2, 10% CO_2, 85% N_2) for 48 h. The cytotoxic effects were evaluated by measuring the diameter of inhibition areola, subtracting for the disk diameter (6 mm).

2.11. Cell culture and MTT assay

Human adherent gastric adenocarcinoma epithelial cells (AGS, ATCC CRL-1739) were cultivated as described by Messing et al. [46]. The cytotoxicity against AGS cells was determined by MTT assay according to Gottesmann et al., [23].

2.12. Bacteria growth and quantitative *in vitro* flow cytometric adhesion assay

H. pylori J99 (ATCC 700824) was cultivated according to Messing et al. [46]. Quantitative *in vitro* flow cytometric adhesion assay were performed as described previously by Messing et al. [46] and Gottesmann et al., [23].

2.13. Statistical analysis

Results are presented as mean value (MV) ± standard deviation (SD). Mean values were compared by a one-way ANOVA test followed by a Tukey's test for multiple comparisons (using GraphPad Prism version 6.0, GraphPad Software, Inc., La Jolla, USA). A p value <0.05 compared to the negative control was considered statistically significant.

3. Results

3.1. Isolation and purification of polysaccharides from *B. alba*

From the fresh stems of *B. alba* (BA), a fresh extract (FE) was obtained by aqueous extraction. Raw polysaccharides (BA1) were isolated from FE by precipitation in ethanol 96% (yield 0.3% *w*/w). From BA1, an AGP fraction (BA2) was isolated with β-D-glucosyl Yariv reagent in a yield of 8.9% (*w*/w), related to BA1 (~ 0.027% referred to the starting material).

3.2. Analytical characterization of AGP BA2

BA2 was analyzed for its carbohydrate content by the resorcinol sulphuric acid assay [40], resulting in 53.07 ± 5.01% carbohydrates in BA2. The concentration of uronic acids in BA2 was determined by the method of Blumenkrantz and Asboe-Hansen, 1973, [41]. Uronic acid content in BA2 was determined with 5.37 ± 0.98% and protein content of 41.56 ± 2.48% in BA2 was quantified by Bradford assay [47].

Approximate content of AGP in the fraction BA1 and in BA2 was determined to be 4.32 ± 0.27% in BA1 and ~ 100% in BA2. The respective results of this test are displayed in the **Supplementary Data Fig. S1**. Table 1 summarizes the overall composition of the AGP BA2.

Table 1. Composition of arabinogalactan protein BA2 from *B. alba.*

Ingredient's content	Yield (%)
Carbohydrate	53.07 ± 5.01
Uronic acid	5.37 ± 0.98
Protein	41.56 ± 2.48
Arabinogalactan protein	~ 100%

Carbohydrate composition of acid-hydrolyzed BA2 was analyzed qualitatively by thin-layer chromatography (TLC) and quantitatively by gas chromatography–mass spectrometry (GC–MS) [36,39,48].

TLC analysis indicated the presence of arabinose, galactose, and galacturonic acid (**Supplementary Data Fig. S2**). Also, GC–MS of the alditol acetates revealed the presence of arabinose and galactose as the main carbohydrates, besides minor amounts of rhamnose, and traces of xylose and glucose. For specific determination of the uronic acids in BA2 the monosaccharide mixture was subjected to high-pressure anion exchange chromatography with pulsed amperometry detection (HPAEC-PAD) [39], which proved the presence of galacturonic acid, being in accordance with the TLC data. Also using HPAEC-PAD, additionally the neutral carbohydrates were analyzed, providing qualitative and quantitative results comparable to those obtained from GC–MS analysis of the alditol acetates. D−/L-configuration of the main monosaccharides of BA2 was determined according to Noe and Freissmuth (1995) [42] after derivatization and separation by capillary zone electrophoresis. The main monosaccharide galactose was found to exist in the D-configuration, while arabinose was L-configurated. The overall sugar composition of the AGP BA2 is summarized in Table 2**.**

Table 2. Carbohydrate composition (mol %) of BA2: neutral sugar composition was determined after TFA hydrolysis by HPAEC-PAD and by analysis of the respective alditol acetates by GC–MS. The presence of galacturonic acid has been determined by HPAEC-PAD and the quantitative amount of uronic acid is based on the assay according Blumenkrantz and Asboe-Hansen, 1973, [41] * D/L configuration were not determined.

Carbohydrate	Amount, determined by HPAEC- PAD [mol %]	Amount, determined by GC–MS [mol %]
Rhamnose*	4	3.6
L-Arabinose	47.9	54
Xylose*	not detectable	1.2
Glucose*	not detectable	1
D-Galactose	42.8	34.9
Galacturonic acid*	5.3	not detectable

As expected for a typical AGP, galactose and arabinose are the main monosaccharides in BA2, accounting for ~90%. In addition, galacturonic acid and rhamnose were found in minor amounts. The results are in accordance with the data obtained from the gel diffusion assay with Yariv reagent, which indicates the presence of AGP in BA2.The presence of galacturonic acid has been determined by HPAEC-PAD and the quantitative amount of uronic acid is based on the assay according Blumenkrantz and Asboe-Hansen, 1973, [41].

For further analysis of the AGP, BA2 was analyzed by SDS-PAGE to confirm purity and to obtain additional information on the respective molecular size. A main protein band was observed in the region >150 kDa (Fig. 1a), smearing from 125 to >250 kDa. AGP are typically known to have a broad molecular weight distribution, as the protein undergoes heavily post-translation modification, especially by a high degree of glycosylation. Fine structures of the glycan moieties can be variable to a certain extent, especially in regard to chain length and degree of substitution of the protein part [49]. In addition, the protein/glycan ratio can vary for the AGP core protein isolated from the same tissue [50]. The migration of glycosylated proteins on the gel and that of the standard proteins are not

well comparable and for that reason, the molecular weight determined by SDS-PAGE is not considered accurate.

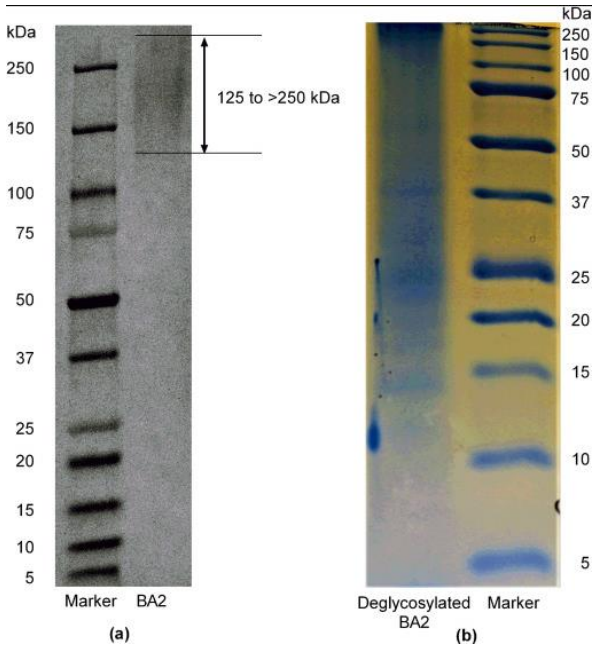


Fig. 1. Gelelectrophoretic investigation of arabinogalactanprotein BA2 from *B. alba*. **(a):** SDS-PAGE (10% polyacrylamide gel) of BA2 stained with Yariv reagent; protein ladder Spectra Multicolor 2 to 250 kDa. Noted that the whole gel was stained with Yariv reagent, but only AGP in BA2 was reacted, the marker was not affected. **(b)**: Tricine SDS-PAGE of BA2 after 24 h deglycosylation by TFMS/anisol; protein ladder SpectraTM multicolor 2 to 250 kDa. Bands at 15 and 25 kDa were exised for further analysis.

After deglycosylation of BA2 the resulting proteins were precipitated in ether/hexane [51] and investigated by Tricine-SDS-PAGE, a protocol that allows separation of proteins and peptides with small size [52], followed by the detection with colloidal Coomassie blue staining (Fig. 1b).

After 24 h incubation with TFMS/anisole, two additional bands appeared at 15 and 25 kDa, and these bands were isolated for further analysis. To obtain a more detailed insight into the primary structure of the isolated bands, the proteins were subjected to tryptic in-gel digestion, and the resulting peptides were analyzed by mass spectrometry using Synapt G2 Si mass spectrometer coupled to nano UPLC. Unambiguous protein identification was however not conclusive for those bands, but rather for BA2. Ribulose biphosphate carboxylase large chain (RBL-BASAL, P25828) was assigned based on data-independent (**Supplementary Table S1**) and target analysis. Fig. 2 illustrates the location of the detected peptides in the protein sequence. An exemplary fragment ion spectrum for peptide TFQGPPHGIQVER is displayed in Fig. 3. It provides good ion coverage and high confidence for the assignment. This peptide was also used for a Blast search and was shown to be unique for RBL of 421 species (respective entries in SwissProt, see examples in **Supplementary Data Table S1**).

Fig. 2. Sequence coverage following data-independent analysis.

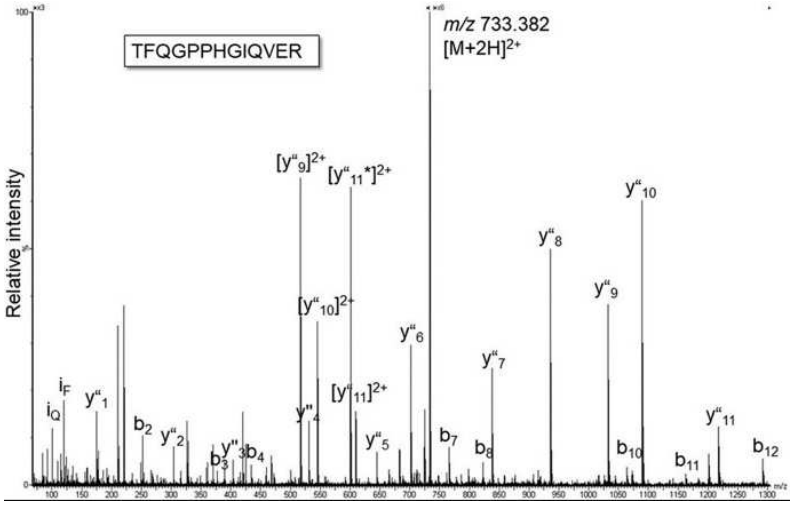


Fig. 3. MS/MS spectrum of *m/z* 733.382 target analysis matched to peptide TFQGPPHGIQVER. Peptide fragment ions of the respective b- and the y-ion series are labelled.

3.3. Influence of FE and RPS on *H. pylori* and human gastric AGS cells

For the investigation of the potential influence of the polysaccharides from *B. alba* on *H. pylori* and human stomach cells, agar diffusion assay against the bacteria and MTT assay on cellular viability of human gastric AGS cells were performed. No effects of FE and RPS BA1 against *H. pylori* in the concentration range from 0.5 to 2 mg/mL were observed within agar diffusion assay. (Note: the authors are aware that agar diffusion assay can provide in some cases false negative results, especially in cases where lipophilic compounds are investigated, which will not diffuse well into the agar matrix. This is definitely not the case for FE and BA2, as these are polysaccharides with high affinity to the agar matrix; therefore, this test system can be used without problems for *B. alba* [53]. FE and RPS B1 (0.1 to 2 mg/mL) did also not have any significant influence within MTT assay on AGS cells over a 24 h incubation protocol (**Supplementary Data Fig. S3**).

As displayed in Fig. 4, the aqueous FE inhibited the bacterial adhesion to AGS cells in a concentration dependent manner, with maximal inhibition at 2 mg/mL (57% bacterial adhesion, corresponding to 43% inhibition). Okra fresh extract [48] served as positive control, which reduced the bacterial

adhesion to the host cells significantly by about 50%. Interestingly, the antiadhesive effects of the RPS turned out to be more pronounced than those of FE (Fig. 4), being concentration dependent and reducing the bacterial adhesion by 67% at 2 mg/mL. These results indicate a potential antiadhesive of the polysaccharides from *B. alba*. Due to very limited amounts of the AGP from *B. alba*, this purified polymer could not be tested on its activity against *H. pylori*.

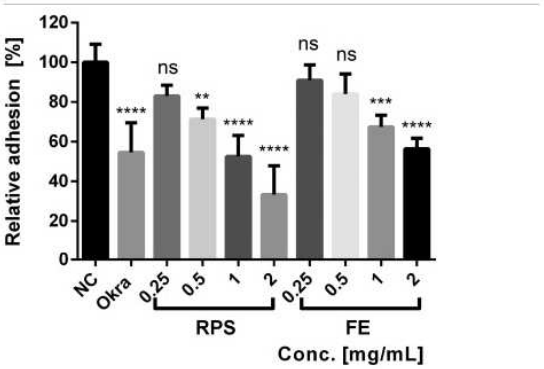


Fig. 4. Relative adhesion (%, related to the untreated control, set as 100%) of labelled *Helicobacter pylori* to AGS cells, after pretreatment with different concentrations of FE and RPS of *Basella alba*. UC: untreated control; Positive control: Okra fresh extract at 1 mg/mL. Values are mean ± SD, $n = 3$ independent experiments with 3 replicates each. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Histological localization of AGP in stems from *B. alba*

Since AGP are hypothesized to act as regulative factor during cell differentiation, tissue development, and somatic embryogenesis [54], it is quite interesting to answer the question on the localization of the AGP in the stem of *B. alba*. Thus, microtome stem sections were prepared and AGP were histochemical visualized by staining and precipitating with β-D-glucosyl Yariv reagent. Yariv reagent is highly specific for interaction with AGP, and is not interacting with arabinogalactans without protein moiety or with non-arabinosylated proteins [55]. Therefore, this reagent can not only be used for identification of AGP in complex matrices, but serves also as a specific and easy to handle staining compound for histological investigations [36]. Fig. 5 shows the transversal section of *B. alba* stem after Yariv staining.

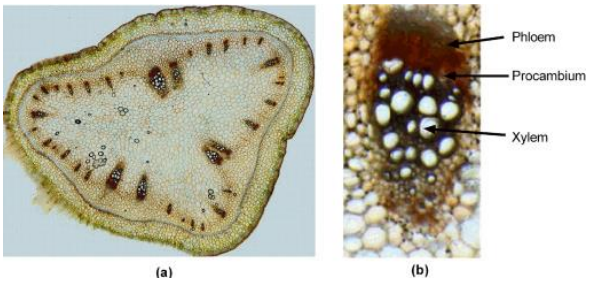


Fig. 5. *Basella alba* stem section, stained with β-D-glucosyl Yariv reagent. **(a)** the whole transversal stem; **(b)** magnification of a vascular bundle from **(a)**.

As expected, the AGP is localized in the stem; highest staining is observed in the vessels, surrounded by a red procambium ring.

4. Discussion

B. alba is widely used as valuable vegetable in Asia, but also as medicinal remedy for a variety of diseases. Due to the high content of mucilage of *B. alba,* functional testing of these polysaccharides

seems a promising subject, as polymeric glycoconjugates have been recognized within the last decade to influence specifically many aspects of cell physiology, especially formation of extracellular matrix [56], interaction with mucins [57,58], cellular proliferation or differentiation [59], induction of human growth factors [60], cellular adhesion or host-pathogen interaction [61].

Cold water extraction of *B. alba* stems revealed about 0.3% of a highly viscous fresh extract, from which about 9% of AGP were isolated by Yariv reagent precipitation. Isolation of AGP by the highly specific Yariv reagent is a simple method, but in general, the amounts of material isolated by this method are quite low. In most cases these amounts are sufficient for analytical characterization of the AGP, but for subsequent experiments for which higher amounts are needed for functional investigations this can cause problems.

Structural features of the so obtained glycoprotein BA2 indicated the presence of a typical acidic AGP, mainly composed of arabinose and galactose and a quite high percentage of protein (about 42%). The broad molecular weight dispersity of BA2 with SDS-PAGE suggests the existence of various glycopeptides. There are two sub-family of AGP designated as “classical” and “non-classical” AGP [62]. Classical AGP possess an N-terminal signal sequence and a region rich in Pro/Hyp, Ala, Ser, and Thr, followed by a C-terminus for the replace of a GPI anchor, which indicates posttranslational modification in mature AGP [49,50]. The protein content in classical AGP is typically lower than 10% with neutral to acidic protein backbones [63]. Protein content in BA2 was found at ~42% and therefore these AGP should be classified as “nonclassical” AGPs.

AGP are involved in many aspects of plant physiology during the growth and development stages including cell fate, cell proliferation, cell expansion, and also protection and nutrition functions [62,[64], [65], [66]]. Many studies have indicated that the oligosaccharide units of glycoconjugates play crucial roles in a broad range of biological processes. These are providing signals for cell surface recognition, but are also providing structural, protective and stabilizing features [67,68]. BA2 was found histochemical localized in the procambium ring of *B. alba*'s stem; this might suggest the function of AGP to mediate cell-cell interactions required for xylem differentiation. This is similar to reports on xylogen, an AGP cluster described for rice (*Oryza sativa* L.) [69], which has been discussed to mediate cell-cell interactions required for xylem differentiation [[70], [71], [72]]. Therefore, these AGP are predominantly accumulated in the meristem, procambium and xylem [72]. From this observation, we hypothesize that the *B. alba* AGP might have functional similarities to this xylogen cluster. This should be confirmed by further studies. As it has been discussed in case of AGP clusters from *Jatropha curcas* embryos, characterized by the occurrence of fascilin, xylogen and LysM proteins with specific actions during seed germination, the high glycosylation of these proteins might lead to a kind so enzyme-silencing during the dormancy period. It is hypothesized that during germination the glycan moieties are enzymatically removed and the protein activity can be started immediately, without prior protein synthesis [36].

The monosaccharide composition of BA2 has been determined by two different analytical methods, namely HPAEC-PAD and GC–MS, with comparable results. HPAEC-PAD seems to be preferable, due to the simple sample preparation and the wide range detection of both neutral and acidic monosaccharides, especially as GC–MS of the alditol acetates will not detect uronic acids. Unfortunately, methylation analysis of BA2 for the unambiguous determination of the carbohydrate linkage of the polymer failed (data not described in results part). We assume that the protein part of the AGP interferes with the permethylation step. It seems necessary to develop validated protocols for efficient linkage analysis of such high protein AGPs. Characterization of the protein part of BA2 by MS assigned ribulose bisphosphate carboxylase/oxygenase or “Rubisco”, an enzyme which is involved in the major step of carbon fixation within the Calvin cycle that helps to store the energy by

photosynthesis [73]. Rubisco is probably the most abundant enzyme on Earth [74] and is the most abundant protein in leaves (accounted for 50% soluble leaf protein in C3 plants [75]). This enzyme is usually present in the chloroplasts of higher plants and consists of two types of protein subunits, the so called eight large subunits (rbcL) of Mw 55,000 Da and eight small subunits (rbcS) of Mw of 14,000 Da [76]. Beside carboxylation, under specific conditions, as *e.g.* strong light, high temperature and low humidity when stomata close and the CO_2 concentration in the leaf decreases, rubisco also provides oxygenation activity [77]. This process is called photorespiration and leads to reduced efficiency of photosynthesis by 25% in C3 plants under no-stresses condition, and up to 50% in stress conditions [78]. Photorespiration is not only a wasteful process, but is also involved in stress protection that could prevent the over-production of the chloroplast stroma under conditions of high light and low CO_2 concentration to prevent water loss [79]. In addition, photorespiratory metabolism might create metabolites like glycine or serine for other processes in plants. Therefore, abolishing photorespiration by engineering rubisco might not necessarily improve the crop yield especially under harsh growth conditions [80]. Since the RBL-containing BA2 AGP from *B. alba* was mostly located at the phloem tissue (detected by Yariv staining), it is suggested that this RBL moiety, after activation by removal of the glycan moiety, might play an important role in the regulation of the photosynthesis and tissue development.

MTT assay and agar diffusion test showed that none of the fractions (fresh extract and RPS) influenced vitality and proliferation of both human AGS cells and *H. pylori*. Such results are important for potential medical applications. These results underline the non-cytotoxic effect of *B. alba* extract against AGS cells, in accordance with previous work that highlighted the non-cytotoxicity of a similar polysaccharide-enriched fresh extract from Okra immature fruits from *A. esculentus* [46]. This is also similar to other polysaccharide preparations used for medical purposes, as *e.g.* fresh extract and polysaccharides from *A. esculentus* [48] or from *Ribes niger* [32]. As AGP from *R. nigrum* has been reported to interact with BabA of *H. pylori* [32], potential influence of FE and BA1 on the adhesion of fluorescent-labelled *H. pylori* to AGS cells was investigated using a flow cytometric adhesion assay [19,46,81]. Interestingly, the polysaccharides form *B. alba* and also FE exerted significant and concentration-dependent inhibition of the bacterial adhesion of *H. pylori* to human host cells under *in vitro* conditions. As in the investigations pre-treatment of the bacteria was performed it can be assumed that the antiadhesive effect is due to an interaction with outer membrane proteins of *H. pylori*, which are responsible for host cell recognition and adhesion. This is similar to carbohydrates investigated in detail as antiadhesive entry blocker against *H. pylori*, *e.g.* sialyl-lactose or mucilage from immature Okra fruits [46]. The observed effects with BA1 and FE are similar to data obtained from mucilage from Okra's fresh extract and pectic rhamnogalacturonans isolated during bioassay-guided fractionation from *A. esculentus* extract [46]. This result suggested that the high molecular weight polysaccharides from *B. alba* might block OMPs of the bacteria, leading to ineffective colonization of *H. pylori* to gastric cells. Interestingly, the high uronic acid content of the raw polysaccharide is like that observed for other antiadhesive polysaccharides, described as antiadhesive polymers against the adhesion of *H. pylori*. It has also to be kept in mind that *H. pylori* has also high binding affinity to gastric mucus, which bears strong negative charge even under the acidic pH of the gastric environment due to terminal sialic acid residues and sulphate [82]. Gastric mucins again are binding glycoproteins for *H. pylori*, which interacts mainly with mucins *via* SabA, which recognizes the sialic acid residues in the gastric mucus. It is speculated that also the quite viscous and negatively charged *B. alba* polysaccharides mimic gastric mucins, leading to interaction and binding to SabA. The antiadhesive effects of negatively charged polymers, especially sialic acid-containing compounds has been reviewed recently in [83] and interestingly the borders between classical medical therapy by use of registered drugs and the tremendous development of functional foods are getting weaker and

weaker, as has been shown by strongly acidic sialic acid-derived carbohydrates for *H. pylori* infections [83,84]. Unfortunately, exogenous sialic acid containing oligosaccharide 3'-sialyl-lactose with significant *in vitro* antiadhesive activity against *H. pylori* failed within double-blind, placebo-controlled clinical study, probably due to instability reasons in the stomach [85]. For such practical reasons the use of non-digestible, pectin-like polymers has the advantage of high chemical stability in the intestinal environment, the easy way of preparation from biological sources and in most cases also concerning the absence of toxicity [86]. Therefore, mucilage containing plant materials could serve as an effective source of antiadhesive polysaccharides, in case these polymers are strongly negatively charged, soluble in water, and stable in the intestinal environment. All these aspects are fulfilled for rhamnogalacturonans from immature fruits of *A. esculentus* (Okra pods). These polysaccharides have been characterized in detail for the respective structural features [23,46,48] and also the molecular interaction with BabA and SabA has been described in detail. Additionally, the traditional use of Okra hot water extracts in the Asian and African traditional medicine for treatment of gastritis and stomach infection could be a hint for clinical evidence. Interestingly, the mucilage from *B. alba*, widely used in Vietnam traditional medicine for the same use, is obvious, leading to the here presented study. The presented data highlights on one side the structural aspects of the polysaccharides from *B. alba* but reflects also a potential antiadhesive effect of the mucilage-containing extract against *H. pylori*. The here presented functional data might be interpreted as a first pilot study to pinpoint a potential antiadhesive activity, but more detailed studies must be performed as follow-up, especially to identify the potential binding partner on the bacterial surface (which are in most cases described for antiadhesive polysaccharides either BabA or SabA), but also to clarify the structural interaction between both binding partners. The use of *B. alba*'s stem as food products could establish preventive strategies against the stomach infection caused by *H. pylori*, but further clinical studies must be initiated to rationalize this traditional use.

On one hand, antiadhesive carbohydrates could be a new and innovative way to interact with *H. pylori*, but still some limitations might occur during the development of such compounds for clinical use. The first aspect to be investigated in further studies is the stability of the polymers within the gastric environment, especially concerning potential degradation or denaturation. This might be not a great problem, as polysaccharides are normally not degraded by the proteases in the stomach. On the other side, denaturing phenomenon under acidic conditions are known for polysaccharides, leading to strongly reduced solubility. Further, it must be investigated if the interaction of the antiadhesive agent with the surface structure of *H. pylori* will also occur under acidic conditions of the stomach. Similar studies have been performed with structurally defined rhamnogalacturonans, indicating that low pH does not reduce the antiadhesive effects of the polymers [46,48]. Additionally, it must investigated if the *B. alba* polysaccharides after oral ingestion are able to penetrate the endogenous mucin layer of the stomach, to get in close contact with the bacteria, which normally are not free-floating in the stomach, but are more or less hidden in the mucin layers or are adhering to the membrane of the epithelial cells. For this, the antiadhesive compounds need to be mucin-interacting or better mucin-penetrating polymers. This has been shown recently for antiadhesive pectins [23] which exert a high affinity to stomach mucin. No data are available of such properties can also be observed for AGP or other *B. alba* polysaccharides. On the other side antiadhesive compounds without mucin-binding capacity can easily be formulated in liposomes, coated with mucin-directing pectins, which target the antiadhesive compounds to the mucin layer. Additionally, antibiotic cargo can also be inserted into this antiadhesive nanoparticles to ensure elimination of the bacteria when the polysaccharide has docked on the outside of the bacterium [22,23]. From these points, the present study with *B. alba* polysaccharides must be seen as a pilot report for first identification of the antiadhesive

properties of these compounds against *H. pylori*, but many follow-up studies should be initiated to identify the potential and the use of these compounds.

5. Conclusions

We have reported for the first time the antiadhesive activity against *H. pylori* attachment toward AGS cells of several polymers extracted from an aqueous suspension of fresh stem of *B. alba*. The carbohydrate (β-1,3/1,6 galactan with arabinose side chains, as typical for AGP) and protein part of AGP in this plant has been characterized for sugar composition and peptide sequences, and the protein part has been identified for the first time as a cluster of ribulose bisphosphate carboxylase that might play an important role in regulating the photosynthesis and tissue development. We hypothesized that the high molecular weight polysaccharides possessing high content of arabinogalactan backbone and small amounts of galacturonic could act as antiadhesive agents against *H. pylori* attachment to the stomach host cells. The specific polymers in *B. alba* RPS might block the major adhesins BabA and SabA in *H. pylori* leading to the reduction of attachment to host cells. These hypotheses need to be verified experimentally. In addition, once the correlations between monosaccharide composition in RPS and the degree of esterification, methylation and acetylation are elucidated, the structural requirements for an antiadhesive activity will be achieved. To the end, the results obtained in this study has opened a promising prospective for the discovery of natural products from plants that block bacterial adhesins and hence prevent the further adherence to the host cells. On the other side, future investigations should be performed to elucidate the clinical potential of *B. alba* extracts for *H. pylori* infection. From traditional use this plant has been used for a long time to cure gastritis, and the present data indicate relevant *in vitro* anti-adhesive effects of the polysaccharides against the adherence of *H. pylori* to gastric epithelia. *In vitro – in vivo* correlation should pave the way to the future use of an easy-to-handle *Basella* extract for *H. pylori* treatment, which can also be seen as a cheap and effective way of treatment in low-income countries.

Author contributions

HTN and AH conceived and designed the experiments. HTN performed the experiments, analyzed the data, and wrote the first draft of the manuscript. FH helped during performing of histological investigations. SK performed MS protein analysis and revised and discussed the manuscript. FMG and AH supervised the research, interpreted results, reviewed the first draft, and improved the final manuscript.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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