RESEARCH ARTICLE

Peptides from *Pisum sativum* L. enzymatic protein digest with anti-adhesive activity against *Helicobacter pylori*: Structure–activity and inhibitory activity against BabA, SabA, HpaA and a fibronectin-binding adhesin

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Scope: Identification of anti-adhesive peptides against *Helicobacter pylori* obtained by enzymatic hydrolysis of seed proteins from *Pisum sativum L.* (Fabaceae).
Methods and results: Bioassay-guided fractionation of protein tryptic digest by ultrafiltration, size exclusion chromatography (SEC) and reversed phase chromatography (RPC) were used. Identification of bioactive peptides was achieved by MALDI-TOF-MS. Adhesion of *H. pylori* was monitored by two different assays, using a quantitative in vitro assay on human AGS cells with evaluation of bacterial binding by flow cytometry, beside a semi-quantitative in situ adhesion assay using FITC-labelled *H. pylori* on human stomach tissue sections. From two highly active fractions (F3, F3.3) two anti-adhesive peptides (S3, S5) were identified. Neither F3 nor S3 or S5 had any cytotoxic effect against *H. pylori*. By hemagglutination assay and semiquantitative dot blot overlay assay with immobilized ligands it was shown that F3 interacts specifically with *H. pylori* adhesins BabA, SabA, HpaA and a fibronectin-binding adhesin, while S3 and S5 inhibit only BabA. It was demonstrated that BabA, usually interacting with carbohydrate motifs such as fucosylated blood group antigens, interacts with the peptide moieties.
Conclusion: Bioactive peptides from pea protein could be applied as functional ingredients for protecting infants and children against infections such as *H. pylori*.

Keywords:
Adhesion / BabA / *Helicobacter pylori* / *Pisum sativum* L. / SabA

1 Introduction

Infections with *Helicobacter pylori*, one of the main pathogens for gastric ulcers [1], and a group I carcinogen [2] are mainly acquired in early childhood, during the first 2–5 years of life [3, 4]. By the age of 10, overall prevalence is more than 75% in developing countries, whereas only 10% are infected in developed countries, but prevalence can rise to 30–40% in children from lower socio-economic groups [5]. The *H. pylori* transmission is discussed to occur by oral–oral, gastro-oral (*via* emesis), faecal-oral modes, and also by drinking water supply (in developing countries) [4, 6].

With regard to problems of treatment by antibiotics and prophylaxis by vaccination [4, 7] (risk of resistance and shift to especially pathogenic strains, selection of proper antigens), the adhesion, and therefore the infectionof *H. pylori* to the gastric mucosa should be prevented, ideally by dietary oral intervention. Without specific bacterial adhesion, the risk of infection and inflammation resulting in gastritis or

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Abbreviations: CHCA, alpha-cyano-4-hydroxycinnamic acid; **HA,** hemagglutination; **OMP,** outer membrane protein; **PVDF,** polyvinylidene fluoride; **RPC,** RP chromatography

possibly in cancer can be minimized. Therefore, research for identification and development of potential anti-adhesive compounds was intensified in the last years [8–12]. The use of such substances can be rationalized for an application directly after an antibiotic eradication treatment of patients in order to minimize the risk of a re-infection.

The potential infection prophylaxis against *H. pylori* by breastfeeding and the finding that it may have long-term protective effects against chronic *H. pylori* infections and reduces the risk of gastric carcinoma [13] initiated the research on potential anti-adhesive proteins and peptides in breast milk. Glycosylated human κ-casein effectively inhibits the adhesion of *H. pylori* to human gastric surface mucous cells [14, 15]. This anti-adhesive effect can be attributed to the fucosylated oligosaccharide moiety [15]. Another study showed that a lower prevalence of *H. pylori* infections in women was related to the presence of the Lewis^b blood group determinant, which is characterized by a di-fucosylated oligosaccharide epitope [16]. Fucosylated structures constitute the major part of neutral oligosaccharides identified in breast milk [17]. The enzymatic release of bioactive peptides from food protein sources other than human milk might offer an alternative way to mimic such functional properties of human milk compounds [18].

Besides milk proteins, different plant proteins from soy, pea, rice, *etc.* are traditionally used in human nutrition. Within the present work, the potential of pea protein as source for bioactive peptides was investigated. Pea protein isolate was enzymatically digested to crude peptides mixtures, from which specific anti-adhesive peptides were identified after bioassay-guided fractionation as inhibitors of *H. pylori*.

In the past years several outer membrane proteins (OMPs), associated or involved to *H. pylori* adhesion to gastric epithelial cells, have been identified. For instance, a fucosylated oligosaccharide structure present in H-1 and Lewis^b blood group antigens (blood group O phenotype) was identified as a receptor motif for the OMP BabA, the blood group antigen adhesin [19, 20].

Further, antigens like sialyl-Lewis^a and sialyl-Lewis^x, which are reported to be predominantly expressed in inflamed gastric tissue, interact with the OMP SabA [21–23]. Such fucosylated and sialylated antigens favour the colonization of *H. pylori* to the gastric mucosa and might even promote the chronicity of infection once gastritis is established [22]. Further, SabA is also reported for polymorphic binding to sialylated glycans, *e.g.* presented on the erythrocyte membrane, suggesting that SabA is the responsible hemagglutinin for the sialic acid-dependent hemagglutination (HA) [24].

Additionally, the glycoprotein fetuin and 3′-sialyllactose are known to inhibit specifically HpaA, a subunit of the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin and in the case of 3′-sialyllactose even reversing HA and detaching *H. pylori* from gastric cells [25–27].

Other interactions with bacterial adhesins are associated with eukaryotic receptors like extracellular matrix proteins. Here, proteins such as laminin, fibronectin and type IV collagen have been proposed to function as receptors for *H. pylori* in the gastric region [28–31].

Regarding these interactions, an array was established using immobilized glycoproteins and neoglycoproteins with binding properties to specific bacterial adhesins [23, 32]. With this tool, the potential mechanisms of anti-adhesive peptides from pea protein digests against *H. pylori* can be more clearly defined and associated to specific bacterial adhesins.

2 Materials and methods

2.1 Materials

3′-Sialyllactose (NeuAcα_{2-3}Galβ_{1-4}Glc) and FITC were purchased from Sigma Chemicals (St. Louis, MO, USA). Pea protein isolate (Nutralys® F85F, 84% w/w protein) was provided by Roquette Frères (Lestrem, France). Synthetic peptides were manufactured and purchased from Thermo Fisher Scientific (Ulm, Germany); identity of synthetic peptides was confirmed by MS. All other chemicals and reagents were purchased from Merck (Darmstadt, Germany) in analytical quality. Fresh Okra fruitextract was prepared as described [9].

2.2 Pea protein enzymatic hydrolysis

Pea protein isolate (50 g) was dissolved in 1.5 L of distilled water at 50°C. Hydrolysis was started by adding 0.56 g of food-grade pancreatic trypsin Novo PTN 6.0S (Novozymes A/S, Bagsvaerd, Denmark) to yield a 1:89 w/w enzyme to substrate ratio. The pH was constantly monitored and controlled at 7.0 through the addition of 1 M NaOH. The reaction was allowed to continue for 2 h. The process was stopped by heat inactivation of the enzyme at 85°C for 5 min. Precipitated material was pelleted by centrifugation at $3800 \times g$ for 20 min at 20°C and the supernatant ultrafiltered with a plate-and-frame device using a 700 cm^2 10 kDa NMWCO PES membrane (UltranLab, Schleicher&Schuell, Dassel, Germany). The retentate was lyophilized and used for further separation of the pea peptides.

2.3 Pea peptides fractionation and purification procedures

The 10 kDa retentate was fractionated by size-exclusion chromatography using a 42×5.0 cm id column packed with Toyopearl® HW-50S (Tosoh Bioscience GmbH, Stuttgart, Germany). Ammonium hydrogen carbonate, 0.1 M, containing 2% v/v 2-propanol, was used as mobile phase. The column was operated at a flow rate of 4 mL/min at 4°C. Eluting compounds were monitored at UV 220 nm,

fractions collected in 7 mL intervals and pooled to yield four major fractions according to Fig. 1, namely F1 to F4. The fractions were lyophilized and tested in the *H. pylori* adhesion assay.

Further purification of F3, being most active in the functional assay, was achieved by RP chromatography (RPC) using a 11.8 × 1.0 cm id column packed with Amberchrom® CG-161S resin (Tosoh Bioscience GmbH). Mobile phases used were 0.1% v/v TFA in distilled water (A) and 0.1% v/v TFA in 2-propanol (B). Elution was accomplished with a linear gradient starting from 5% B to 60% B in 7 column volumes, flow rate 0.85 mL/min, and UV 220 nm. Fractions of 4 mL each were collected and pooled to obtain three main fractions: F3.1 to F3.3. The fractions were lyophilized before further analysis. An additional control sample was generated by re-uniting these three main fractions after RPC to account for potential loss of activity during fractionation.

2.4 Mass spectrometric characterization and identification of different peptides in pea hydrolyzate fractions

The molecular weights of peptides in the pea hydrolyzate fractions were determined by MALDI-MS. Amino acid sequence delineation of anti-adhesive pea peptides was achieved by subsequent MALDI-TOF-TOF MS/MS analysis of major ion peaks identified by a previous MALDI-MS analysis.

Selection of anti-adhesive pea peptides for subsequent amino acid sequence determination by MS/MS analysis was done as follows: Fractions with a minimum of 25% inhibition of *H. pylori* adhesion or higher were regarded as active anti-adhesive fractions. Complex MALDI-MS spectra of these active fractions were then compared with MS spectra obtained from inactive fractions. The latter fractions served as negative controls. Mass peaks that occurred exclusively in the active fractions or had only minor relative peak-intensities (< 20% of basepeak) in the negative controls, but high relative peak intensities (< 45% of basepeak) in the active fractions, were chosen for subsequent MS/MS-based amino acid sequencing. Only m/z values in the range between 800 and 3500 were considered.

MALDI-TOF sample preparation was done by mixing equal volumes of 0.1 mg/mL aqueous pea peptide fraction with α-cyano-4-hydroxycinnamic acid (CHCA) on a stainless steel MALDI-target. The concentration of alpha-cyano-4-hydroxycinnamic acid was 4 mg/mL. The solvent of CHCA was 50% ACN containing 0.1% TFA. The sample-CHCA mixtures were allowed to dry at room temperature on the MALDI-target. Proper co-crystallization of sample and CHCA MALDI-matrix was controlled utilizing a microscope with 40-fold magnification.

The employed mass spectrometer was a 4800 MALDI-TOF-TOF instrument (Applied Biosystems, Darmstadt, Germany), equipped with a NdYAG-Laser emitting laser light at a wavelength of 355 nm. A laser-intensity setting of about 3300 arbitray units was chosen to achieve optimum signal-to-noise ratio. Two thousand laser shots *per* sample (50 shots at 40 different positions in the preparation) were collected and averaged. The instrument was operated in positive reflector ion mode using an acceleration voltage of

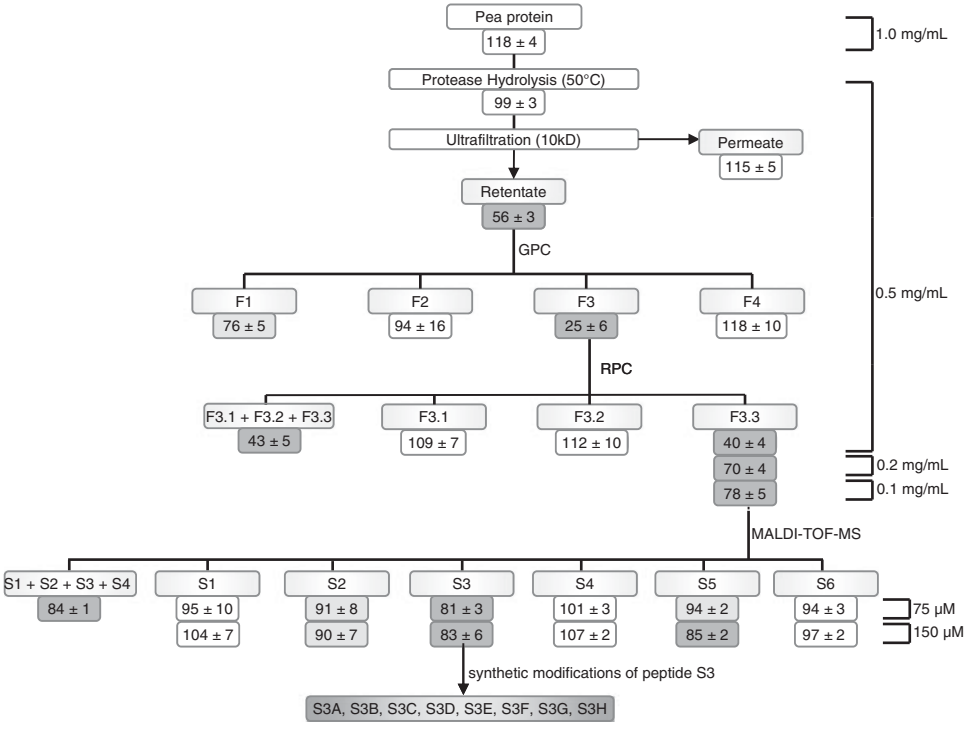


Figure 1. Bioassay-guided fractionation of peptides, obtained by tryptic digest of pea protein. Peptides S1 to S6 were identified by MALDI-TOF from fraction F3.3 and manufactured synthetically for activity testing. Adhesion activities are expressed as the mean adhesion (±SEM, $n = 3$ from independent experiments) of FITC-labelled *H. pylori* to AGS cells within the flow cytometric assay at 1.0 and 0.5 mg/mL (fractions) and at 75 µM as well as 150 µM for the peptides. Data are related to the untreated control of *H. pylori* (= 100%).

20 kV. Mass accuracy was equal to or better than 50 ppm in default calibration mode. A mass accuracy of equal to or better than 5 ppm was achieved by using internal calibration of mass spectra.

MS/MS data were acquired manually and automatically in positive ion mode utilizing high-energy collision-induced dissociation with air at 1 kV. Two thousand and eight hundred laser shots *per* sample (70 shots at 40 different positions in the preparation) were collected and averaged. Default calibration was applied to calibrate MS/MS spectra. Laser-intensity settings were 4550 arbitrary units.

Pea peptide amino acid sequences were assigned to MS/MS data using the ProteinPilot™ software suite (Applied Biosystems/MDS Sciex) with the integrated Paragon™ algorithm [33]. The following parameters where chosen to enable the amino acid assignment to MALDI-MS/MS-data by the Paragon™ algorithm: sample type: identification; cystein alkylation: none; digestion: trypsin; instrument: 4800; special factors: none; species: no restriction; ID-focus: biological modifications; database: Uniprot/Swiss-Prot (version January 23, 2007, including a contaminants database, both in the FASTA-format); search effort: thorough.

2.5 Bacteria and growth conditions

Helicobacter pylori ATCC 700824 (J99, identification for quality control by PCR for vacA, cacA genes) was cultivated for two or three passages to minimize the risk of phase-variable switching of OMP genes. *H. pylori* was therefore incubated for 48 h under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2, CampyGen™ Oxoid) at 37°C on Tryptic Soy Agar (Becton Dickinson, Germany), supplemented with 5% defibrinated sheep blood (Oxoid, UK).

2.6 Cell culture

Human adherent gastric adenocarcinoma epithelial cells (AGS, ATCC CRL-1730) were kindly provided by Professor Dr. med. Winfried Beil (Medizinische Hochschule Hannover, Germany) and were grown in RPMI 1640 with L-glutamine (PAA, Germany), supplemented with 10% FCS, on tissue culture flasks (75 cm^2, Sarstedt, USA) and 6-well plates (Sarstedt, USA) in 5% CO_2.

2.7 Labelling of bacteria

Agar grown *H. pylori* were harvested and resuspended in sterile carbonate buffer (pH 9.0). The OD of the suspension was photometrical measured at 550 nm and 1.0×10^8 bacteria were resuspended in 1 mL of sterile buffer [34]. Ten microlitre of a FITC solution (1% in DMSO) were added and incubated with the bacteria for 45 min. Fluorescent labelling was terminated by pelleting the bacteria ($3150 \times g$, 5 min). Bacteria were washed twice in PBS to remove excess FITC and were gently resuspended for further use.

Preliminary studies were performed to determine labelling conditions and to prove that the fluorescence was maintained during the course of co-incubation experiments with epithelial cells.

2.8 *Helicobacter pylori* adhesion assays

In vitro testing on anti-adhesive activity of test compounds against *H. pylori* on AGS cells was accomplished by a flow cytometric method [35]. *In situ* experiments with histological sections of human gastric mucosa and FITC-labelled *H. pylori* were performed according to [9] and evaluated by fluorescent microscopy. Multiple paraffin-embedded tissue sections from human gastric mucosa were from the antrum mucosa of *H. pylori*-negative individuals, which showed no major pathologic alteration. In principle, the adhesion of FITC-labelled *H. pylori* to the epithelium was assessed by fluorescent microscopy and imaging [9, 11]. The quantity of adhering bacteria to the epithelial surface was evaluated under double-blinded conditions. Maximal adhesion was expressed as total 100% adhesion with a score (+++++). Lower adhesion was indicated by (++++, +++, ++, +, −) with missing (−) adhesion being found in the positive control (okra fresh extract, according [9]). Additionally, the fluorescence area intensity was calculated by ImageJ® software (Olympus, Germany), standardizing the fluorescent area of the negative control as 100%.

2.9 HA assay

Erythrocyte suspension (2%) from fresh human EDTA-blood (blood group O^+) in PBS was prepared. Fresh agar grown *H. pylori* (48 h) were harvested and resuspended in PBS. Before incubating with erythrocytes, bacteria were pretreated with test compounds and PBS as untreated control (OD 2.0). Suspensions were incubated for 2 h in the dark, followed by three times washing with PBS-Tween®20 and resuspension in 250 µL PBS. Fifty microlitre of these solutions were transferred to 96-well plates, followed by serial dilution in PBS. Fifty microlitre of the erythrocyte suspension was added to each well. The plate was gently shaken for 5 min and incubated for 1 h at room temperature. HA was measured by MicroWin® 2000/4.38 (Microtek Labsystems, Overrath, Germany). As positive control, the total fraction of acidic human milk oligosaccharides at 1.0 mg/mL was used (Danone Research – Centre for Specialised Nutrition, Friedrichsdorf, Germany). The fraction of acidic human milk oligosaccharides includes a variety of structures bearing α-2,3-linked sialic acids capable of inhibiting the adhesins SabA and HpaA [17, 36].

2.10 Dot blot overlay assay

The assay was based on previously described methods [23, 32]. Untreated polyvinylidene fluoride (PVDF) membranes (Biotrans™ PVDF membrane, 0.2 μm pore size; MP Biomedicals, Germany) were spotted with 2 μL of a solution, containing 1 μg of glycoproteins and neoglycoproteins. Spotted compounds were as following: laminin (from human placenta, Sigma Chemicals), fibronectin (Biochrom AG, Berlin, Germany), Lewis^b-HSA, H1-HSA, sialyl-Lewis^a-HSA, 3′-Sialyllactose-HSA and 6′-Sialyllactose-HSA (IsoSep AB, Lund, Sweden). HSA (fraction V, Sigma Chemicals) and BSA (fraction V, immunoglobulin free, protease-free, Sigma Chemicals) were included as negative (nonglycosylated) controls.

Bacteria with an OD of 6.0 in PBS were fluorescein labelled by incubation for 30 min at room temperature, with FITC in a concentration of 100 μg/mL. Bacteria were washed three times, resuspended in PBS and incubated with test compounds (OD 0.25, 2 h) in the dark at room temperature.

PVDF membranes were blocked in TBS (20 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6) containing 5% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ for 2 h at room temperature. Labelled and pre-treated bacteria were recovered by centrifugation at 2700 × *g* for 5 min, washed three times with PBS and resuspended in 1 mL blocking buffer (final OD = 0.5). Membranes were overlaid with the bacterial suspensions and incubated for 30 min at 4°C in the dark without mixing. They were washed three times at room temperature for 5 min on a rotary shaker in TBS containing 0.05% Tween®20, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. The fluorescence of adherent pre-treated and untreated bacteria was detected by a Typhoon imaging system (Typhoon 9200, Amersham Biosciences, Freiburg, Germany).

2.11 Statistical analysis

Statistical tests were performed by using SPSS®. The experimental results were expressed as the mean ± SD. Data were assessed by analysis of variance. In case the analysis indicated significant differences between group means, each group was compared by Dunnett's *t*-test (two-sided), and $p < 0.05$ was considered to be statistically significant.

3 Results

For investigation of tissue-specific adherence and potential anti-adhesive activities of pea peptides, two different test assays were applied: for quantitative evaluation and mechanistic studies, an *in vitro* system with AGS cells was used; adherence of FITC-labelled *H. pylori* to AGS cells was measured by flow cytometry [35]. Additionally, a semi-quantitative *in situ* adhesion system, based on gastric tissue sections from human biopsies, was used to confirm results of this *in vitro* test [9].

Pea protein in itself did not exhibit any anti-adhesive activity against *H. pylori*. The protein was subjected to trypsin digestion for 2 h (Fig. 1). The resulting hydrolyzate obtained after heat inactivation and removal of the enzyme showed significant inhibition of bacterial adherence, which clearly indicates that the release of peptides from native proteins can change dramatically the functionality of the product. The crude hydrolyzate was subjected to ultrafiltration using a 10-kDa membrane. Anti-adhesive activity (56% bacterial adhesion) was found within the retentate fraction at 500 μg/mL. Within bioassay-guided fractionation, an even increased activity (25% adhesion) was found after size-exclusion chromatography in fraction F3 while the other fractions were less active or even inactive (see Supporting Information). Subsequently, F3 was subjected to RPC (see Supporting Information). Three major subfractions were obtained, from which F3.3 was found to be the active fraction, inhibiting the bacterial adhesion of *H. pylori* to AGS cells by 60% at 500 μg/mL and 22% at 100 μg/mL level. In contrast, F3.1 and F3.2 were inactive. The unification of the three fractions (F3.1+F3.2+F3.3) indicates that peptides from F3.1 and F3.2 do not enhance synergistically the activity of F3.3, since testing showed an adhesion of *H. pylori* to AGS cells of 43%. For unambiguous identification of active peptides, the fractions F3.1, F3.2 and F3.3 were investigated by MALDI-TOF-TOF MS/MS analysis. Signals of peptides present in the active fractions but not found in the inactive mixtures were specifically selected for subsequent MS/MS-based amino acid sequencing. Six peptide sequences (S1 to S6) were determined by this method (Fig. 2, sequences given in Table 1) and unambiguously identified with the ProteinPilot™ software suite (Applied Biosystems) as peptide fragments from pea Legumin A or A2 protein. No signs for posttranslational modification of the peptides were found. Subsequently, the respective peptides were synthesized for further unambiguous testing on anti-adhesive properties. The undecapeptide S3 was found to be the most active compound reducing the bacterial adhesion of *H. pylori* significantly to 81 and 83%, respectively (75 resp. 150 μM corresponding to 0.1 resp. 0.2 mg/mL). The anti-adhesive effect of pea protein peptides, if measured in a 75 μM mixture of the four peptides S1 to S4, did not show any significant changes in activity (84%) compared to S3, indicating the absence of any synergistic effects. Therefore, peptides S3 and S5, which inhibited adhesion by 6–17% (75 resp. 150 μM), were assessed two of the active peptides obtained from pea protein tryptic digest.

For confirmation of these results, fraction F3, as well as peptide S3, were investigated on anti-adhesive activity in the *in situ* assay on human gastric tissue (Fig. 3). *H. pylori*, pre-treated with fraction F3, showed a strongly diminished adhesion in this test system (about 70% inhibition). Also, S3

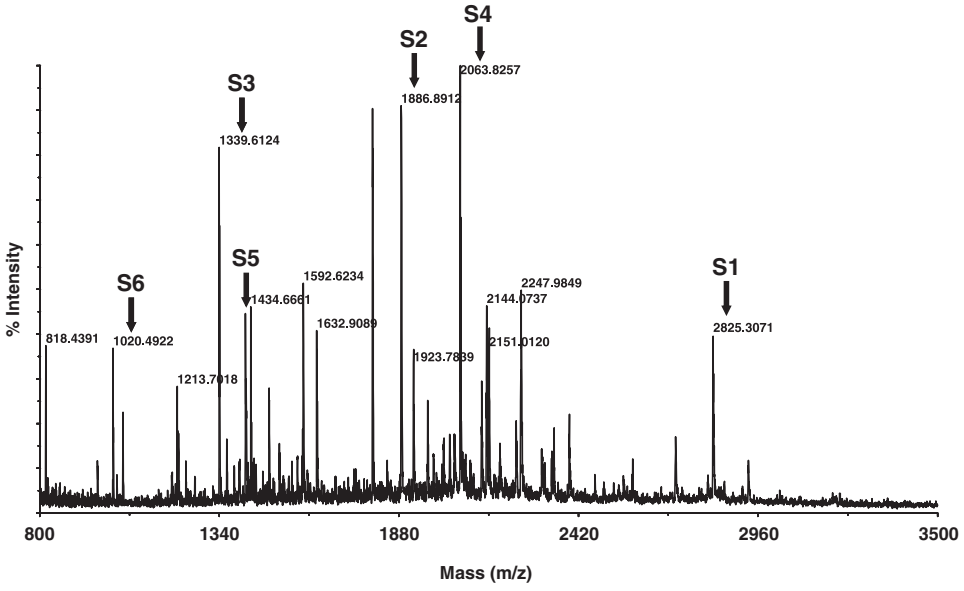


Figure 2. Total ion chromatogram from the MALDI-MS experiments of anti-adhesive fraction F3.3 and peptides S1 to S6 exclusively present in the active fraction F3.3 but absent in inactive fractions F3.1 and F3.2.

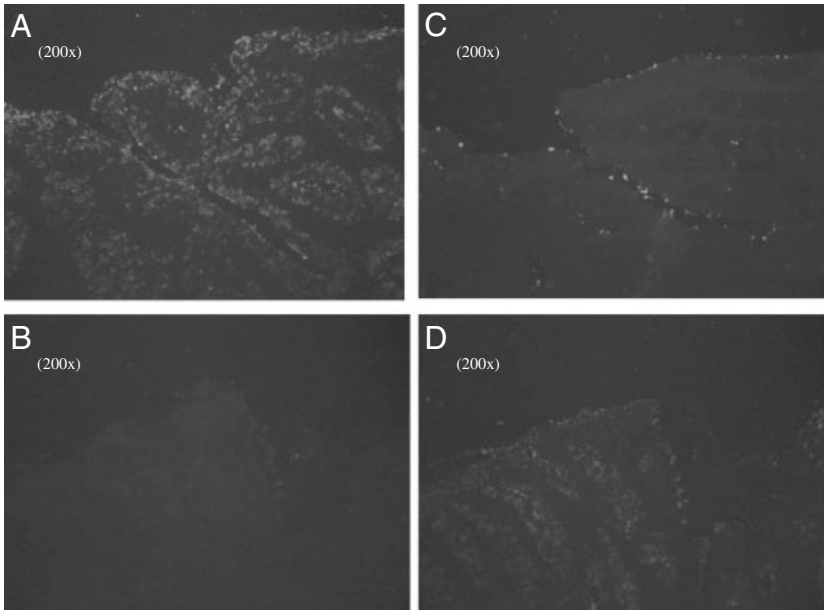
Table 1. Sequences of peptides S1 to S6 (as revealed by high-energy CID MALDI-MS/MS), from synthetic peptides S3A to S3H, with the mean adhesion (±SEM) of FITC-labelled *H. pylori* to AGS cells after pre-treatment of the bacteria with the respective peptides

Test compound	Amino acid sequence	Rel. adhesion in (%) (±SEM; $n = 3$)	
		75 μM	150 μM
S1	Leu-Asp-Ala-Leu-Glu-Pro-Asp-Asn-Arg-Ile-Glu-Ser-Glu-Gly-Gly-Leu-Ile-Glu-Thr-Trp-Asn-Pro-Asn-Asn-Lys	95±10	104±7
S2	Leu-Asn-Ile-Gly-Pro-Ser-Ser-Ser-Pro-Asp-Ile-Tyr-Asn-Pro-Glu-Ala-Gly-Arg	93±8	94±6
S3	Asp-Phe-Leu-Glu-Asp-Ala-Phe-Asn-Val-Asn-Arg	81±3	83±6
S4	Trp-Glu-Arg-Glu-Glu-Asp-Glu-Glu-Gln-Val-Asp-Glu-Glu-Trp-Arg	98±2	107±2
S5	Glu-Leu-Ala-Phe-Pro-Gly-Ser-Ala-Gln-Glu-Val-Asp-Arg	94±2	85±2
S6	Gly-Asp-Phe-Glu-Leu-Val-Gly-Gln-Arg	94±3	97±2
S3A	Asp-Phe-Leu-Glu-Asp	102±3	108±6
S3B	Ala-Phe-Asn-Val-Asn-Arg	97±4	104±1
S3C	Leu-Glu-Asp-Ala-Phe-Asn-Val-Asn-Arg	95±5	100±4
S3D	Asp-Ala-Phe-Asn-Val-Asn-Arg	89±4	89±3
S3E	Asp-Phe-Leu-Glu-Asp-Ala-Phe-Asn-Val	91±4	91±4
S3F	Asp-Phe-Leu-Glu-Asp-Ala-Phe	85±3	90±5
S3G	Leu-Glu-Asp-Ala-Phe-Asn-Val	93±2	94±3
S3H	Asp-Ala-Phe	104±6	105±1
Untreated control	–	100±2	100±2
Positive control	–	70±5	70±5

Data are related to the untreated control of *H. pylori* (= 100%). Positive control: 3′-sialyllactose (15 mM).

reduced the bacterial adhesion strongly (about 40% inhibition). These data clearly confirm the anti-adhesive activity of F3 and S3. On the other side, the bioassay-guided fractionation indicated also a kind of non-linear activity profiling: the anti-adhesive effects were not steadily increasing during the peptide purification steps, but were getting less for the individual peptides isolated and tested. This is a clear indication for the presence of other active compounds in the mixture, influencing the anti-adhesive properties of the mixture in a synergistic way.

For evaluation of structure–activity relation of undecapeptide S3, eight different fragments of S3 (S3A to S3H) were synthesized in order to obtain more information on a potential peptide motif responsible for anti-adhesive activity (sequences Table 1). None of these short-chain peptides (5–9 amino acids) showed activities comparable to those of S3. While peptides S3A to S3C and S3H were completely inactive, peptides S3D to S3G showed in general minor inhibition activities ranging from 15 to 6%. Concerning the respective sequences, no peptide consensus motif was found



H. pylori pre-treated with	concentration	Blinded fluorescence microscopy evaluation *	rel. fluorescence area (%)
(A) untreated control	-	+ + + + +	100
(B) Okra (positive control)	Fresh extract	+	4
(C) fraction F3	1.0 mg/mL	+ +	51
(D) peptide S 3	300 μM	+ + +	71

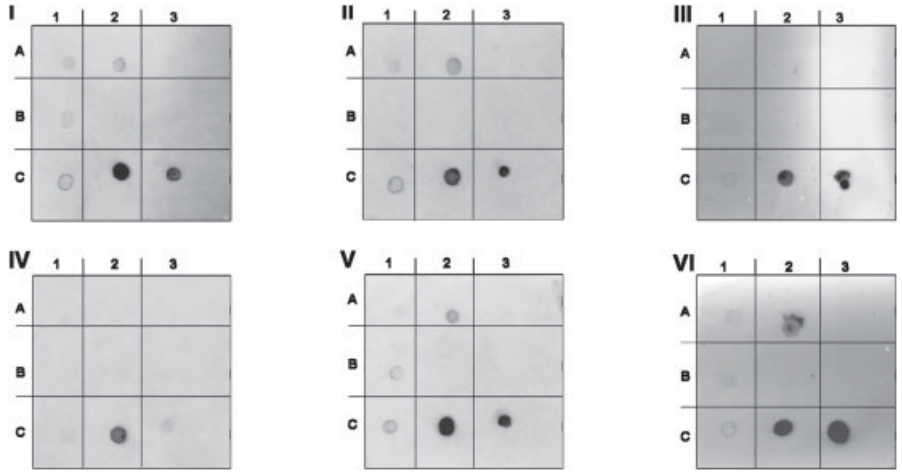
Figure 3. Representative fluorescence microscopy images of FITC-labelled *H. pylori* adhering to human gastric mucosa sections: untreated *H. pylori*control (A), *H. pylori* pretreated with Okra FE (positive control) (B), peptide fraction F3 at 1 mg/mL (C) and peptide S3 (300 μM) (D). Images are equalized in brightness and fluorescence intensity and assessed by double blinded microscopic evaluation as well as by fluorescent imaging by ImageJ® software; *+++++: strong adhesion, +: weak adhesion.

to be clearly responsible for the anti-adhesive activity. This proposes the assumption that probably the entire peptide sequence of 11 amino acids as found in S3 is necessary for full anti-adhesive activity. On the other side, the existence of certain secondary structure and three-dimensional peptide folding can change functionality of peptides. For that, preliminary conformational modelling experiments, with the molecular operating environment software, of the different peptides indicated a high degree of folding of the undecapeptide S3 which was clearly absent in the low molecular peptide fragments S3A to S3H (data not shown).

For further evaluation of the mode of action, a potential direct cytotoxicity of the active fractions and peptides (0.1–0.5 mg/mL) against *H. pylori* was tested by agar diffusion assay against amoxicillin as positive control. Bacterial growth was not influenced by any of the test compounds (data not shown). Therefore, the anti-adhesive effect was probably due to interaction with OMP.

For pinpointing the respective bacterial adhesins blocked by the active peptides, a semi-quantitative dot blot overlay assay was implemented. Therefore, putative ligands, known to interact specifically with *H. pylori* adhesins, were immobilized by spotting on PVDF membranes. A representative selection of typical ligands for *H. pylori* adhesins used for these experiments were Lewis b and H type I blood group antigen conjugates, interacting with the OMP BabA, 3′-sialyllactose interacting specifically with the OMP HpaA, sialyl-Lewis a and laminin known for interacting with the OMP SabA, and fibronectin with a not yet determined bacterial adhesin affinity. Further, HSA and BSA were used as controls to exclude non-specific binding of *H. pylori* to spotted compounds on the membrane. In addition, 6′-sialyllactose was used to demonstrate the binding specificity of HpaA to 3′-sialyllactose.

For the respective binding assays, *H. pylori* were preincubated with the test compounds, followed by FITC labelling. The membranes, containing the immobilized glycoproteins and neoglycoproteins, were incubated with the bacteria. Non-adhering bacteria were washed of and adhering *H. pylori* were visualized by imaging. As expected, significant bacterial adhesion to the immobilized ligands was obvious (Fig. 4). As positive controls, bacteria were preincubated with 3′-sialyllactose, interacting specifically with the HpaA adhesin. As expected no adhesion to spotted 3′-sialyllactose-HSA conjugate was observed (Fig. 4II). Additionally, a mixture of human milk oligosaccharides, containing, for example, α2,3-linked sialic acids and fucosylated oligosaccharides present in the Lewis^b or H-type I blood group antigen binding epitopes, was used at 1.0 mg/mL in the function of a second positive control [17, 36]. Since a various amount of oligosaccharides in human



A1: Lewis b-HSA
A2: H type I-HSA
A3: Human serum albumin (HSA)
B1: 3`-Sialyllactose-HSA
B2: 6'-Sialyllctose-HSA
B3: Bovine serum albumine (BSA)
C1: Sialyl-Lewis a-HSA
C2: Laminin
C3: Fibronectin

Figure 4. Adhesion of pre-treated FITC-labelled *H. pylori* wild type (wt) strain J99 to immobilized ligands: (I) untreated control and pre-treated bacteria with (II) 3′-sialyllactose, (III) total acidic oligosaccharides from human milk, (IV) fraction F3, (V) peptide S3 and (VI) peptide S5. (Neo)glycoproteins spotted on PVDF membranes (1 μg *per* spot) were overlaid with FITC-labelled *H. pylori* and adherent bacteria detected by a fluorescence scanner. The locations of spotted (neo)glycoproteins are indicated below.

Table 2. Effects of pre-treated *H. pylori* with fraction F3, peptides S3 and S4 on the agglutination of human erythrocytes

Test compound	Concentration	Reduction of titre
Positive control	1.0 mg/mL	2.5±0.4
Positive control	0.5 mg/mL	1.9±0.3
Raw pea protein hydrolyzate	0.5 mg/mL	1.3±0.3
Raw pea protein hydrolyzate	0.1 mg/mL	0.5±0
Fraction F3	0.5 mg/mL	2.8±0.3
Fraction F3	0.1 mg/mL	0.9±0.3
Peptide S3	150 μM	1.0±0.3
Peptide S3	75 μM	0.8±0.3
Peptide S4	150 μM	0
Peptide S4	75 μM	0

Values from three independent experiments indicate the reduction of the logarithmic titres as mean MW±σ related to untreated control. Positive control: human milk oligosaccharides, acidic fraction [17, 36].

milk have an affinity to adhesins like BabA, HpaA and SabA, it was obvious that pre-treated *H. pylori* would be strongly influenced in the interaction to these three OMPs (Fig. 4III).

In cases of pre-treatment of *H. pylori* with F3, a strong reduction of bacterial interaction with Lewis^b-, H type I- and 3′-sialyllactose-HSA conjugates as well as with fibronectin was observed (Fig 4IV). F3 also affected, to a smaller degree, the binding to sialyl-Lewis^a conjugate, but not to spotted laminin. These finding clearly indicate that F3 interacts specifically with *H. pylori* adhesins BabA, HpaA, fibronectin-binding adhesion and in less a stronger way also with SabA.

In contrast, peptides S3 and S5 exhibited only inhibition of Lewis^b-HSA mediated adhesion, suggesting inhibition of the BabA adhesin, while other adhesins were not significantly influenced (Fig. 4V and VI).

The inhibiting activity of F3 against the sialic acid binding adhesin (SabA) should additionally be confirmed in a simple HA assay (Table 2): *H. pylori* was pre-treated with test compounds and was incubated after a serial dilution together with human erythrocytes. In the case of bacteria pre-treated with F3, a significant inhibition of HA was observed. The minor reduction of agglutination by peptide S3 at 150 μM (1.0±0.3) also reflects a possible weak interaction of this peptide with *H. pylori* SabA, even if this interaction was not visualized by the respective dot blot arrays.

In summary, complex fractions F3 inhibits in a multifunctional way *H. pylori* adhesins, acting against at least four different OMPs, while the undecapeptide S3 and peptide S5 are mainly interacting with the OMP BabA.

4 Discussion

In the past years, many natural anti-adhesive compounds against *H. pylori* adhesion have been identified [10–12, 37, 38]. Nevertheless, none of these compounds was described with an affinity to specific OMPs. Thereby, this study aimed to identify new highly active compounds from natural sources and define specific interactions with selected bacterial adhesins.

Peas are part of a common diet in most countries as an important alternative source of protein with a long history and are available in large quantities. Pea protein from was selected as a potential source for bioactive peptides, since preliminary studies showed promising ways to inhibit

H. pylori adhesion by mimicry of functional properties of human milks compounds (unpublished results).

Within a bioassay-guided fractionation different complex fractions and several peptides were identified with strong anti-adhesive activities. It was obvious that the purified peptides, *e.g.* undecapeptide S3, were significantly less active than the more complex fractions, which is due to the monovalent inhibition of a single bacterial adhesin by the purified peptide. In contrast, the use of a complex heterogenic mixture is able to interact with *H. pylori* OMPs in a multi-target strategy and therefore lead to a blocking of several proteins, responsible for the bacterial adhesion. This clearly demonstrates that in the scenario of different relevant OMPs for an adhesion process, complex mixtures can possibly be used much more effective than highly purified single compounds.

For a structure activity relation of the active undecaptide S3 various fragments, differing in lengths were synthesized (S3A to S3H, Table 1). Functional testing demonstrated a general loss of inhibitory strength compared to the native peptide S3. Only peptides S3D, E, F and G slightly blocked adhesion (about 10% reduction). If compared to S3 amino acids sequence, a homologous motif with the terminal and integral tripeptide sequence, namely Asp-Ala-Phe, can be identified. Hence, the synthesized tripetide (Asp-Ala-Phe) S3H was tested and shown to be inactive, which possibly means that this sequence can only be functionalized, if integrated into a longer peptide chain. We assume that most probably the entire peptide sequence of at least eleven amino acids as found in S3 is necessary for its inhibitory activity to the bacterial adhesin, due to a possible formation of three-dimensional peptide folding, necessary for interactions with the receptor target.

The specificity of the active peptides towards *H. pylori* OMPs was shown by dot blot glycoconjugate assay. Within these experiments, purified fraction F3 showed affinity to BabA, HpaA, SabA and an unknown fibronectin binding adhesin. S3 and S5 interacted with BabA, as shown by the clear reduction of intensity in the interaction between labelled pre-treated *H. pylori* and spotted Lewis^b-HSA conjugate (Fig. 4V and VI). If regarding the interaction of the same bacteria with the H-type I blood group antigen, it seems that there is no inhibitory effect induced by S3 or S5. This indicates most probably a selective specificity of these peptides to BabA, since the interaction of *H. pylori* to the binding epitope of H-type I blood group antigen remains unaffected.

Only in presence of a complex peptide mixture as in fraction F3, a strong blocking of the interaction between *H. pylori* and the BabA receptor blood group antigens Lewis^b and H type I was observed. Additionally, fraction F3 prevented completely the interactions of *H. pylori* with 3′-sialyllactose and also with fibronectin. Concerning the binding of *H. pylori* to fibronectin, experiments have shown that this interaction was abolished rather by denaturation than by deglycosylation of glycoproteins, proposing that the affinity of this unidentified surface structure depends on the recognition of unknown protein moieties [23]. Since fraction F3 is composed of a complex peptide mixture, which inhibits the fibronectin interaction to the *H. pylori* adhesin, this potentially implies structural similarities between pea peptide and fibronectin motifs. Additionally, it confirms the assessed hypothesis that interactions of *H. pylori* to fibronectin rely on peptide motifs.

Further, the interaction of bacteria with the spotted sialyl-Lewis^a conjugate was also demonstrated to be reduced in contrary to laminin. In conclusion, these inhibitions suggest either a strong mimicry or additional binding sites of peptides from F3 to known receptor structures of BabA, HpaA, SabA and to a not yet identified adhesin with affinity to the ECM protein fibronectin.

Interactions of *H. pylori* with sialyl-Lewis^a and laminin, both associated with binding to SabA, were not strongly affected by bacterial pretreatment with F3 and S3 in blot assays. Still, if comparing in detail the intensity of untreated and F3-pretreated bacteria adhering to the spotted sialyl-Lewis^a, a reduction in intensity induced by F3 is obvious. To confirm this potential interaction of peptides with SabA, a HA assay was performed. Here, fraction F3 intensely inhibited the HA (2.8 titre levels), reaching even higher activity compared to an acidic human milk oligosaccharides fraction (positive control; 2.5 titre levels), known to include sialic acid-linked structures [17, 36].

Initially, hemagglutinating activity of *H. pylori* to human erythrocytes was suggested to be dependent on sialic acid [25]. NeuAcα2-3Gal (3′-Sialyllactose) was characterized as the sialic acid binding epitope since it could competitively inhibit HA [25]. In this process, *N*-acetyl-neuraminyl-lactose-binding hemagglutinin was identified and denoted as the *H. pylori* adhesin A (HpaA) [28]. Later, these results were questioned and the SabA adhesin shown to be the *H. pylori* hemagglutinin. Furthermore, erythrocyte cell surface mapping demonstrated as preferred binding sites for SabA extended ganglioside glycans [28].

Considering this, the HA inhibitory activity of peptides from F3 suggests a potential interaction with the SabA-mediated adhesion. It also confirms the diminished binding of *H. pylori* to immobilized sialyl-Lewis^a-HSA conjugate.

By these results, it can be assumed that the adhesins BabA, SabA and HpaA, known to interact with carbohydrate ligands, as, *e.g.* fucosylated blood group antigens and sialic acid structures, are also capable to interact with peptide moieties. Which recognition region of the bacterial adhesins has affinity to the inhibitory exogenous peptides needs further elucidation.

With the bioassay-guided fractionation approach described in the present article starting from a crude protein followed by a complex peptide mixture and ending with the isolation and characterization of single anti-adhesive peptides, whose functionalities were confirmed by their synthetic counterparts, we paved the way for establishing the possibility of a quality control for the complex

peptide mixture by MS. The quality can be checked by the presence or absence of the specific peptide sequences and can even be extended to quantify the peptides using multiple reaction monitoring and stable isotope labelled standards.

Thus, enzymatically hydrolyzed pea protein can be a promising tool for mimicking functional properties of human receptor structures to prevent *H. pylori* adhesion to gastric epithelial cells. The practical use as a functional food additive to prevent primary infection or even secondary recurrent infection of patients after antibiotic eradication therapy will be worth investigating by future dietary intervention studies. On the other side, it should be clarified if oral consumption of peas can lead to the *in vivo* formation of these anti-adhesive peptides directly in the stomach. In case such peptide formation occurs, epidemiological investigations should indicate a decreased infection rate of the human collectives against *H. pylori*. Besides, pea protein is traditionally used in human nutrition not only for adults but also for toddlers and children. Hence, bioactive peptides from pea protein could be applied as functional ingredients for protecting infants and children against infections such as *H. pylori*.

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