First characterization of bioactive components in soybean tempe that protect human and animal intestinal cells against enterotoxigenic Escherichia coli (ETEC) infection

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A first characterization of bioactive components in soya bean tempe that protect human and animal intestinal cells against ETEC infection.

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Abstract

Tempe extracts can inhibit the adhesion of enterotoxigenic *Escherichia coli* (ETEC) to intestinal cells and thereby can play a role in controlling ETEC induced diarrhoea. The component responsible for this adhesion inhibition activity is still unknown. In this research we describe the purification and partial characterization of this bioactive component of tempe. After heating, defatting and protease treatment, the extracts were found to remain active. However, after treatment with polysaccharide degrading enzyme mixtures the bioactivity was lost. Ultrafiltration revealed the active component to be larger than 30 kDa. Further purification of the bioactive tempe extracts yielded an active fraction with an increased carbohydrate content of higher arabinose content than the non-active fractions.

In conclusion, the bioactive component contains arabinose and originates from the arabinan or arabinogalactan side chain of the pectic cell wall polysaccharides of the soya beans. Which is probably released or formed during fermentation by enzymatic modifications.

Keywords

Bioactivity, adhesion, soybeans, fermentation, polysaccharides, *E. coli*, tempeh, diarrhoea

Introduction

Diarrhoeal diseases of humans and farm animals are frequently caused by infection with Enterotoxigenic *Escherichia coli* (ETEC). ETEC strains are associated with two major human clinical syndromes: Weanling diarrhoea among children in developing countries and traveller's diarrhoea (*1, 2*). ETEC is also an important and global cause of severe, watery diarrhoea in the offspring of some animal species such as newborn calves and suckling and weaned pigs (*3*).

Adhesion of ETEC to intestinal epithelium is known to be a prerequisite for colonization and infection of the intestinal tract. Adhesion of ETEC onto the small intestinal microvilli does not cause significant morphological changes, but it is a requirement for the secretion of enterotoxins that alter the behaviour of the enterocytes by increasing secretion and/or reducing absorption (*3*).

The adhesion of ETEC bacteria to intestinal epithelial cells can be mediated by (proteinaceous) fimbriae. These fimbriae bind to carbohydrate-specific receptors (binding sites) at the epithelial cell surface. Inhibition of adhesion can be due to carbohydrate epitopes that are structurally similar to the binding sites of the epithelial cells and, therefore, adhere to the bacteria and act by competitive inhibition. Non-adhering bacteria are subsequently removed by regular bowel cleansing mechanisms (*4-6*). Human breast milk oligosaccharides are known to act as these so-called anti-adhesins (*7*).

Other studies concluded that proteinaceous component(s) released from lactic acid bacteria decrease the adhesion of ETEC to piglet ileal mucus (*8*). Also, bifidobacteria are known to secrete a proteinaceous component that prevents the adhesion of ETEC to intestinal epithelial cell lines (*9*). This adhesion inhibition can be due to competitive exclusion between bacteria, steric hindrance or chemical changes of adhesion receptors of the epithelial cells.

Tempe is a traditional, fermented soya bean product made from dehulled, soaked and cooked soya beans inoculated with mould, usually a *Rhizopus* spp. During the fermentation mycelial biomass is formed and the fungal enzymes break down the soya bean macromolecules. This contributes to the development of a desirable texture and flavour in the product. It also inactivates or eliminates some soya anti-nutritional components (*10*).

Carbohydrases are used in piglet diets to improve nutrient utilization and it has been reported that the addition of certain carbohydrases to piglet diets reduced the frequency and severity of diarrhoea (*11*). Other research showed that the hydrolysis products of non-starch polysaccharides of soya bean meal are beneficial in fluid balance during ETEC infection (*12*). The enzymatic activity during fermentation could give the same beneficial effects to the soya beans.

Earlier research indicated that the severity of diarrhoea in ETEC induced weaned piglets was less when these piglets were fed on tempe instead of toasted soya beans (*13*). Kiers et al. (2007) investigated the effect of tempe on fluid absorption in piglets and suggested that a water-soluble, high molecular mass fraction (> 5kDa) of tempe is able to protect against fluid losses induced by ETEC. As a consequence, this fraction could thus play a role in controlling ETEC induced diarrhoea (*14*). Furthermore, tempe extracts can inhibit the adhesion of ETEC to intestinal epithelial cells in both piglet brush border cells (*15*) and human epithelial (Caco-2) cells (*16*). Tempe extracts were found to interact with ETEC bacteria and could possibly form a layer coating (part of) the bacterial surface, which may contribute to the observed decrease of ETEC adhesion to intestinal epithelial cells (*16*).

In this research we describe the characterization of the bioactive component(s) in tempe, which inhibit adhesion of ETEC to intestinal cells.

Material and methods

Materials

Dehulled yellow seeded soya beans (*Glycine max*) were supplied by Kleinjan V.O.F. (Rhoon, The Netherlands). Malt extract agar (MEA) and peptone, were purchased from Oxoid (Basingstoke, UK). Brain heart infusion broth (BHI) was purchased from Becton Dickinson (Sparks, MD). Enzymes were obtained from various sources as described in Table 1. All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

Tempe preparation

Dehulled, full-fat yellow-seeded soya beans were soaked overnight in tap water using three cycles of accelerated acidification at 30ºC (*17*). Next, the beans were washed with tap water and cooked in fresh tap water for 20 min in a bean:water ratio of 1:3 (v/v). Subsequently, the beans were cooled and superficially dried at room temperature on perforated trays. For the fungal inoculation of the soya beans a sporangiospore suspension was used. The suspension was prepared by scraping off the sporangia from pure slant cultures of *Rhizopus microsporus* var. *microsporus* (LU573) grown on MEA for 7 days at 30ºC, and suspending them in sterile distilled water with 0.85% (w/v) NaCl and 0.1% (w/v) peptone (PPS). After inoculation of the cooked beans with the sporangiospore suspension (10 mL kg^{-1} corresponding to an initial inoculum level of 10^6 CFU g^{-1} beans). Viable sporangiospores of *R. microsporus* were enumerated as colony forming units as described previously (*18*). The beans (450 g) were packed into hard-plastic, perforated boxes (205 x 90 x 45 mm) and incubated for 72 h. Fermented soya beans (tempe) were freeze-dried and milled (Ultra Centrifugal Mill ZM 200, Retsch GmbH, Haan, Germany) using a 0.5 mm sieve and stored at -20ºC until further processing.

Bioactivity assay

Brush borders were isolated from epithelial cells of the intestine of early weaned pigs as described before (*19*). The concentration of brush border was between 10^5-10^6 brush border mL^{-1} in phosphate buffered saline pH 7.2 (PBS). The brush borders were tested for adhesion of ETEC K88 strain ID 1000 and *E. coli* (O149:K91) strain ID1084 (source: ID-Lelystad, Lelystad, The Netherlands). The ETEC K88-positive (ID 1000) and K88-negatieve strain (ID1084) were grown overnight at 37ºC in BHI. The cultures were centrifuged (10 min, 3000 *g*, 20°C) and washed twice in PBS, followed by suspending the pellets in PBS to an optical density of 0.75 correlating with a concentration of 10^9 CFU mL^{-1}.

Freeze-dried fractions of tempe extracts (10 mg) were suspended in 1 mL PBS (1 w/v %), mixed for 1 h and centrifuged (10 min, 10000 *g*, 20ºC). Dilutions of the supernatants (0.25 and 0.1 w/v %) were prepared in PBS. Aliquots (30 µL) of each of the three solutions were mixed with 30 µL of bacterial suspension and 30 µL of brush borders and incubated at room temperature with continuous gentle shaking for 1 h. The number of bacteria adhering to 12 brush borders, each derived from a single epithelial cell, was determined by phase contrast microscopy. The percentage adhesion was calculated as the average number of ETEC K88 per brush border relative to a PBS control.

Preparation of fraction "active 1"

The tempe extracts were characterized by measuring the bioactivity after applying different treatments, i.e. defatting, protease treatment, heating and filtration. Defatting was performed by three consecutive additions of hexane (1:10 (w/v) tempe: hexane) followed by filtration through a folded paper filter (Schleicher & Schuell GmbH, Dassel, Germany) and air-dried, all at 20ºC. Tempe extracts were prepared by suspending 75 g of defatted tempe powder in 1 L of distilled water and stirring for 1 h at room temperature, while the pH was adjusted to pH

8.0 with 2 M NaOH every 30 min. The soluble tempe material was obtained by collection of the supernatant after centrifugation (30 min, 25000 *g*, 20ºC). The supernatant was filtered through a folded paper filter followed by a second centrifugation step.

Heat treatment was done by heating the extract for 15 min at 100°C. For protease enzyme treatment, proteinase K or pronase E were added (0.01% w/v) and incubated overnight at room temperature, followed by heating for 10 min in order to inactivate the enzymes. Then, the extracts were filtered using centrifugal filters with a membrane cut-off of 30 kDa (Centriprep YM-30, Millipore, Billerica, MA). Both filtrates and retentates were freeze-dried and used for the bioactivity assay. Also combinations of treatments were performed (see Figure 2 for combinations of treatments) and tested for bioactivity. Finally, the so called fraction "active 1" was obtained by a combination of defatting, filtration, proteinase K treatment and heating. Figure 1 shows the order of treatments to obtain fraction "active 1".

Enzyme treatments of fraction "active 1"

Freeze-dried fraction "active 1" (10 mg mL^{-1}) was solubilised in 20 mM NaOAc-buffer (pH 5.0). Powdered enzymes (10 mg mL^{-1}) were solubilised in NaOAc buffer (pH 5.0). Carbohydrases (Table 1) were added (20 µL) each to 1 mL of fraction "active 1" solution and the mixture was incubated overnight at room temperature (20°C) followed by heating for 10 min at 100°C.

After enzyme incubations, the samples were tested for bioactivity and carbohydrate molecular weight distributions were determined. The molecular weight distribution was determined by high performance size exclusion chromatography (HPSEC) using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA). Freeze-dried fractions were dissolved in 0.2 M sodium nitrate (5 mg mL^{-1}) and injected onto three TSK Gel columns in series (superAW 2500, superAW 3000, superAW4000, each 6 mm × 150 mm, Tosohaas, Stuttgart, Germany) in

combination with a superAW Lguard column (Tosohaas). Elution took place at 30 °C with 0.2 M sodium nitrate at 0.6 mL min^{-1}. The eluate was monitored using a refractive index (RI) detector (RI61, Shodex, New York, NY). The system was calibrated with pullulan standards (mass range 180-4.04*10^5 kDa).

Next, the enzyme-treated samples were dialysed using 3 kDa filters (Microcon YM-3 3,000 NMWL, Millipore) to remove the degradation products. Monosaccharide composition of the remaining part was determined after hydrolysis using combined methanolysis/trifluoroacetic acid (TFA) hydrolysis. Sample solutions (100 µL; 1 mg mL^{-1}) were dried using a vacuum oven at 40ºC during 4 h. Next, 1.0 mL of 2 M HCl in dry methanol was added, flushed with nitrogen and closed. The samples were incubated for 16 h at 80ºC, followed by cooling and evaporated under a stream of dry air at 40ºC. Subsequently, 0.5 mL of 2 M TFA was added and the samples were heat treated for 1 h at 121 °C in screw-capped glass vials in a heating block, followed by evaporation under a stream of dry air at 40ºC. The remaining material was washed twice with methanol followed by evaporation and finally dissolved in 1 ml of distilled water and diluted 10 times.

The monosaccharide composition was determined by high performance anion exchange chromatography (HPAEC) using a Dionex ICS 3000 system (Dionex, Sunnyvale, CA) connected with a PAD-detector (Dionex ICS3000). A CarboPac PA-1 column (2*250mm, Dionex) in combination with a CarboPac PA guard column (2*50 mm, Dionex). Samples were injected (20 µL) onto the column and eluted for 30 min with millipore water at a flow rate of 0.3 mL min^{-1}, followed by a 15 min linear gradient of 0-40% (v/v) 1 M NaOAc in 0.1 M NaOH, continued by 5 min elution with 1 M NaOAc in 0.1 M NaOH, followed by 5 min elution with 0.1 M NaOH. Finally the column was equilibrated with millipore water for 25 min. As the post-column mobile phase 0.5 M NaOH was used, to allow a proper detection, with a flow rate of 0.1 mL min^{-1}. A standard was made with 1 mg mL^{-1} fucose, rhamnose,

arabinose, xylose, galactose, glucose, mannose, glucuronic acid and galacturonic acid and treated with the same procedure as the samples.

Preparation of fraction "active 2"

For isolation of the bio-active substance in tempe, freeze-dried tempe powder (200 g) was suspended in 2 L of 35 mM sodium phosphate buffer (pH 8.0) and stirred for 1 h at room temperature (20°C), while the pH was adjusted every 30 min. The insoluble parts were removed by centrifugation (30 min, 25000 *g*, 20ºC) followed by filtration of the supernatant through a folded paper filter and a second centrifugation step.

Size exclusion chromatography was performed using an Akta explorer system (GE Healthcare, Uppsala, Sweden). The supernatant (200 mL) was applied onto a Superdex 200 PG column (4.5 L, fractionation range 1×10^4–6×10^5 Da, GE Healthcare) and eluted with 35 mM sodium phosphate buffer pH (8.0) at a flow rate of 40 mL min^{-1}. The eluate was monitored at 210 and 280 nm and fractions (210 mL) were collected. Five runs were performed and fractions eluting at the same time were pooled. Pooled fractions were dialysed against distilled water with a 12-14 kDa dialyzing tube (Medicell international, London, UK) and freeze-dried. Freeze-dried fractions were tested for bioactivity.

To purify the active part, the active freeze-dried fractions that eluted between 1.9-2.9 L were pooled and solibilized in 250 mL Millipore water. The solution was again applied onto the Superdex 200 PG and fractions (200 mL) were collected. The fractions were dialysed, freeze-dried and tested for bioactivity. The active fractions were pooled and further described as fraction "active 2".

Fraction "active 2" (10 mg mL^{-1}) was dissolved in 35 mM sodium phosphate buffer (pH 6.0). Anion exchange was performed using a glass filter (diameter 25mm) filled with 3 mL of the resin (DEAE CL 6B, GE Healthcare) on top of a vacuum erlenmeyer. First, 3 mL 35 mM

sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl was eluted, to maximize exchangeable counter ions, followed by washing of the resin with 15 mL 35 mM phosphate buffer (pH 6.0). Next, 3 mL of fraction "active 2" solution was added. The unbound fraction was collected and 3.5 mL 35 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl was added to the resin and used to elute the bound material. Finally, 1M NaOH was added to clean the resin and remove the so-called strongly bound fraction, which was immediately adjusted to pH 6.0 with HCl. The unbound, bound and strongly bound fractions were dialyzed, freeze-dried, weighed and tested for bioactivity.

The unbound fraction (active 3) was again dissolved in 35 mM sodium phosphate and brought to pH 3.5 with formic acid and eluted through the cationic resin SP Sepharose Fast Forward (GE Healthcare) with the same solutions as described for the anion exchange resin with the exception of using a 35 mM sodium phosphate/formic acid buffer (pH 3.5) instead of the 35 mM sodium phosphate buffer (pH 6.0). The fractions collected were dialyzed, freeze-dried, weighed and tested for bioactivity.

Results

Characterization of the active component in tempe

Different treatments were performed to characterize the bioactive components in tempe. Figure 2 shows the bioactivity of the untreated tempe extracts as well as the extracts after heating (HE), de-fatting (DF), proteinase K and Pronase E treatment (PROK and PROE) and ultrafiltration (<30 or >30). The results show that the activity remained after de-fatting, protease treatment and heating at 100ºC of the extracts. Filtering with a cut-off membrane of 30 kDa showed that all activity was found in the retentate, which contained all material >30 kDa. Various treatments were combined and bioactivity was present after the combination of defatting, protease treatment, heating at 100ºC and ultrafiltration, and is presented as "active

1" in Figure 2. The results on heating and protease treatment suggest that the bioactive component is not proteinaceous.

The monosaccharide composition of the polysaccharides present in fraction "active 1" was determined. This fraction consists for 48 % (w/w) of carbohydrates. The main monosaccharides of this fraction were arabinose, galactose and galacturonic acid as shown in Table 2. Next, fraction "active 1" was treated with various carbohydrate-degrading enzymes (listed in Table 1), which were selected based on the sugar composition (Table 2), and the remaining bioactivity was measured. The results are shown in Figure 3. After treatment with Gammanase (GAM), Pectinex Ultra SP (PEC) and Liq+ (LIQ) the bioactivity was lost. The other enzyme-treated extracts remained bioactive. The enzymatic degradation of the tempe extracts was then evaluated with HPSEC. Figure 4 shows the chromatograms of fraction "active 1" (gray line) and the fractions "active 1" after enzymatic degradation with the enzymes (black lines). The chromatograms of arabinofuranosidase A (ARA-A) and arabinofuranosidase B (ARA-B) are not shown because almost no degradation was observed. The chromatograms show different degradation profiles caused by the various enzymes. The chromatograms of GAM, PEC and LIQ (which caused loss of bioactivity) showed a clear degradation, particularly of material eluted at low elution volumes (between 8-10 min) in the chromatogram (size 65-796 kDa).

Two bioactive samples (treated with endo-galactanase with arabinofuranosidase A and B (GAL+ARA) and driselase (DRI)) and two non-active samples (GAM and PEC) were selected and dialyzed. During dialyses the material <3 kDa, which eluted around 12.5 min, was removed. From the remaining material the sugar composition was determined (Table 2). After treatment with GAM, GAL+ARA and PEC a decrease in total sugars was observed (data not shown), which was expected because of the removal of the mono- and oligosaccharides released during dialysis. After incubation with DRI the polysaccharides were

not degraded to monosaccharides, but to intermediate sized molecules as shown in the chromatogram (Figure 4). After degradation with GAM and PEC the fraction "active 1" lost activity and showed a decrease in arabinose residues from 29.6 to 5.3 and 7.1 mol %, respectively, whereas galactose decreased in all enzyme-treated samples (Table 2). Despite the reduction in galactose, the GAL+ARA and DRI degraded samples remained bioactive. These two samples showed much higher molar proportions of arabinose than the GAM and PEC degraded samples. The molar proportion of galacturonic acid did not show any relation with the decreased activity caused by GAM and PEC. Also, the recovery of individual sugars after enzyme treatment was calculated. The GAM and PEC treatment resulted in a removal of 88 and 93% (w/w) of all arabinose residues present in the extract before the enzyme treatment, respectively (data not shown).

Purification of the active component

The tempe extract was purified (Figure 1) using two steps of size exclusion chromatography, labelled as SEC 1 and SEC 2. Figure 5a shows the SEC elution pattern of tempe extract. In this figure the fractions collected and their corresponding activities are also indicated. The bioactivity was measured for the indicated fractions 1-11, because the active part is >30 kDa. The activity was situated mainly in the intermediate part (fractions 4-6) of the chromatogram. At all three concentrations applied the adhesion was completely inhibited. The activities found are comparable to the activity of the total tempe extract (TTE) applied at 1% (w/v) and these fractions even had a stronger adhesion inhibition capacity at the lower concentrations. This concentration effect was also observed in the fractions that are eluted before or just after the most active fractions. The material of all runs eluting between 1,9 and 2,9 L (intermediate peaks) was pooled and again applied onto the SEC column. The resulting elution pattern with the fractions collected and corresponding bioactivities is shown in Figure 5b. Fractions 4-6

had the highest activities. They were located just before the main peak in the chromatogram, thereafter the inhibition activity decreased quickly.

After SEC 2, the active material eluted between 1.8 and 2.4 L (fractions 4-6) was pooled and denoted as fraction "active 2". This material was eluted through an anion exchange resin column. An unbound, bound and strongly bound fraction were collected, representing 38, 13 and 29% (w/w) of the starting material, respectively. The bioactivity was measured and fraction "active 2" showed an adhesion of 11.1±3.6 % (average ± SEM) at a concentration of 0.1% (w/v) extract. The unbound material showed an adhesion of 14.0±3.8 % (average ± SEM), and the bound and strongly bound fraction 70.0±4.1 % and 99.3±7.5, respectively. Obviously, the bioactive material was present in the unbound material and this part is called "active 3". Subsequently, the fraction "active 3" was applied onto a cation exchange column. Again, an unbound, bound and strongly bound fraction were collected, representing 14, 38 and 32% (w/w) of the material loaded onto the column, respectively. The bioactivity was measured and the highest inhibition activity was found in the unbound material 36.0±5.2 % (average ± SEM) compared with the bound and strongly bound material 84.0±11.5 and 74.0±7.2 %, respectively, all at a concentration of 0.1% (w/v) extract. Since part of the bioactivity was lost by the cationic exchange, we continued our experiments with the bioactive fraction "active 3".

Monosaccharide analysis was performed on fraction "active 3" (Table 3). This fraction contained 59 % (w/w) sugars, which is higher than found in fraction "active 1" (Table 2). Also, it exhibited a higher molar proportion (50%) of arabinose than fraction "active 1". The ratio between arabinose and galactose moieties had changed from 1.2:1 to 2.7:1 for active 1 and active 3, respectively.

Discussion

We examined the chemical component(s) that protect intestinal cells from adhesion of ETEC bacteria. The bioactivity was tested on piglet intestinal brush border cells, but it is also active in inhibiting adhesion to human cells (*16*). The fractions of the first size exclusion chromatographic separation (SEC 1) experiment were also tested on their inhibition capacities to human Caco-2 intestinal cells. Results showed activity in fractions 5 and 6 with an inhibition of the adhesion of ETEC K88 to the intestinal epithelial cells with 60% (no further data shown). Earlier research found an inhibition of adhesion to Caco-2 cells by total tempe extracts of almost 50%, which may possibly reduce ETEC induced diarrhoea in humans (*16*). Since our earlier research (*13, 14*) had shown that the protective effect of tempe against ETEC induced diarrhoea was not diminished by *in vitro* or *in vivo* digestion, we used non-digested tempe extracts in this study for reasons of convenience and to avoid purification difficulties.

After ultafiltration of the tempe extracts the bioactive component(s) was recovered in the >30 kDa fraction. Also, the SEC 1 and SEC 2 experiments showed that the bioactive component(s) is intermediate in size. In the research of Kiers *et al*. (2003) the bioactive component was recovered in the >5 kDa fraction (*13*). They found that high molecular mass (>5kDa) pre-digested and undigested tempe extracts showed equal protection against ETEC induced fluid loss. The HPSEC patterns of these pre-digested and undigested tempe extracts were also identical in the high molecular weight part (*13*).

Tempe contains a diversity of microbial enzyme activities. Proteolytic enzyme activity in the gut could degrade intestinal receptors for ETEC as was shown before for bromelain (*20*). After heating of the tempe extracts the proteolytic enzymes were inactivated (data not shown). Nevertheless, the tempe extracts were still bioactive after heating at 100ºC (Figure 2), thus we concluded that the proteolytic enzyme activity was not responsible for the bioactivity.

Furthermore, it was shown that the bioactive component was not removed by de-fatting the tempe extract and the bioactivity was not influenced by two broad spectrum proteolytic enzymes. Although the fraction "active 1" did not contain carbohydrates only, these findings make it unlikely that the active component was either proteinaceous or lipidic.

Carbohydrates have been described to prevent infection with bacterial pathogens by competitive inhibition (*6*). More specifically, soy polysaccharides have also been shown to reduce the duration of diarrhoea in children (*21*). Previous research on tempe adhesion inhibition indicated that inhibition of adhesion by tempe extracts is caused by an interaction between the ETEC bacteria and the tempe extracts (*16*). The interaction could be caused by adhesion between the fimbriae of the ETEC and carbohydrate epitopes that are structurally similar to the binding sites on the epithelial cells. This strengthens the hypothesis that the bioactive component is made up of carbohydrates.

Tempe contains cell wall polysaccharides from different origins: (1) from soya beans and (2) from the mould *Rhizopus microsporus*. Furthermore, tempe is rich in lactic acid bacteria. The bioactivity of the tempe extracts is enhanced during fermentation (*16*). This can be due to the growth of the mould or lactic acid bacteria or the degradation of macromolecules during fermentation.

Previously, we investigated (*22*) bioactivity of tempe extracts produced with various substrates and the same starter pure culture. All leguminous fermented substrates were bioactive, whereas cereal-derived tempe products (which allowed a similar mycelial growth of *Rhizopus*) showed no bioactivity at all. Also, after inoculation of soya beans with lactic acid bacteria bioactivity was not induced (*22*). These results suggest that the active component is released or formed by enzymatic breakdown from leguminous substrates.

Carbohydrates in soya beans contain mainly cell wall polysaccharides and the small sugars fructose, raffinose and stachyose. These small sugars were removed during soaking, cooking

353 and partly by fermentation of the soya beans and the remaining were removed during
354 filtration of the processing of fraction "active 1". The cell wall polysaccharides of soya are
355 (partly) degraded during fermentation by the enzymes of the mould, which leads to enhanced
356 solubility (*23*). The major carbohydrases of *R. oligosporus* grown on tempe include
357 polygalacturonases, endocellulases, xylanases and arabinanases (*24*). During fermentation the
358 arabinogalactan and pectin fractions are predominantly solubilised (*25*). The major
359 monosaccharide constituents in soya cell walls are galactose, glucose (from cellulose),
360 arabinose and galacturonic acid (*26*). Fraction "active 1" is rich in arabinose, galactose and
361 galacturonic acid, which corresponds to the observation (*25*) of degradation or solubilisation
362 of pectin and arabinogalactan during fermentation.
363 The monosaccharide composition of the remaining polysaccharides in fraction "active 1" after
364 enzyme treatment showed that arabinose is an important component of the bioactive fractions.
365 Enzymes that were able to degrade the arabinose containing polysaccharides were all
366 responsible for a loss of bioactivity, whereas the enzymic removal of galactose and
367 galacturonic acid from the polysaccharides did not specifically cause a loss of activity.
368 After further purification of the active component with SEC and anion exchange (fraction
369 active 3), an increase in molar proportion of the arabinose in the active fraction was also
370 observed, which strengthens this statement. Research published elsewhere (*4, 6, 7*) indicated
371 that sugars other than arabinose may inhibit bacterial adhesion by interaction with the
372 bacteria.
373 Arabinose, next to galactose, is an important monosaccharide in pectic cell wall
374 polysaccharides of soya beans. Arabinose is especially present in the side chains of
375 rhamnogalacturonans. Rather long arabinan side chains with a degree of polymerisation up to
376 30-35, in addition to the (arabino)galactan side chains, have been reported (*27*). It is assumed
377 that some structural epitopes of such arabinans or arabinogalactans are responsible for the

bioactivity in tempe. During fermentation these structures become more soluble and are thereby more accessible to the intestinal cells.

Some commercial carbohydrates (mannose, arabinose, galactose, arabinogalactan, arabinan), were tested, but they were not bioactive (no further data shown).

Since the bioactivity is only found in fractions >30 kDa and is lost after incubation with some enzyme mixtures only a partial degradation of cell wall polysaccharides is needed to obtain the required structure needed for adhesion to ETEC in competition with the binding sites of the epithelial cells.

In conclusion, it is likely that the active component is of carbohydrate nature, containing arabinose. The bioactive component originates from arabinan or arabinogalactan chains of the pectic cell wall polysaccharides of the soya beans and is released or formed during fermentation by enzymatic modifications. Further research to fully characterize the chemical structure of the bioactive component is needed. A full characterization could open new possibilities for producing the bioactive component, probably via the addition of specific enzymes liberating the arabinose containing medium-weight polysaccharides, which makes it a potential ingredient in food or feed matrices.

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Figure captions

Figure 1: Schematic overview of the different treatments performed with the tempe extracts.

Figure 2: Bioactivity of tempe extracts after different treatments.
Concentration of the extracts is 10 mg mL^{-1}. Bars represent average adhesion (n=12) expressed as % adhesion compared to a control without any addition of tempe extracts. Error bars represent standard error of the mean. TTE: total tempe extract, HE: cooked tempe extract, DF: defatted tempe extract, PROK: tempe extract treated with proteinase K, PROE: tempe extract treated with pronase E, >30: tempe extract >30 kDA, <30: tempe extract <30 kDa, Active 1: Combination of DF, >30, PROK and HE.

Figure 3: Bioactivity of fraction "active 1" after treatment with various enzymes.
Concentration of the extracts is 10 mg mL^{-1}. Bars represent average adhesion (n=12) expressed as % adhesion compared to a control without any addition of tempe extracts. Error bars represent standard error of the mean. GAL: Endo-β-1,4-galactanase, ARA-A: Arabinofuranosidase A, ARA-B: arabinofuranosidase B, α-MAN: α-Mannosidase, β-MAN: Endo-β-mannanase, GAM: Gammanase GAL+ARA: Endo-β-1,4-galactanase and Arabinofuranosidase A and B, DRI: Driselase, PEC: Pectinex Ultra SP-L, LIQ: Rapidase Liq+

Figure 4: High performance size exclusion chromatography of tempe extract (gray lines) and tempe treated with various enzymes (black lines).

504 GAL: Endo-β-1,4-galactanase, α-MAN: α-Mannosidase, β-MAN: Endo-β-mannanase ,GAM:
505 Gammanase GAL+ARA: Endo-β-1,4-galactanase and Arabinofuranosidase A and B, DRI: Driselase,
506 PEC: Pectinex Ultra SP-L, LIQ: Rapidase Liq+
507
508 **Figure 5:** Size exclusion elution patterns of SEC 1 and SEC 2 with corresponding bioactivities **A**: SEC
509 1; **B**: SEC 2 .
510 TTE: total tempe extract, The X-axis represent the fraction numbers. The X-as above represents the
511 elution volume. Bioactivity is measured in three concentrations 0.1, 0.25 and 1% (w/v). Bars represent
512 average adhesion (n=12) expressed as % adhesion compared to a control without any addition of
513 tempe extracts. Error bars represent standard error of the mean.

Table 1: Overview of carbohydrases used.

Name	Abbreviation	Main substrate(s)	Source
Endo-β-1,4-galactanase[1,3]	GAL	Galactan	*Asp. niger*
Arabinofuranosidase A[2,3]	ARA-A	Arabinan	*Asp. niger*
Arabinofuranosidase B[2,3]	ARA-B	Arabinan	*Asp. niger*
α-Mannosidase[4]	α-MAN	Mannan	Jack beans
Endo-β-mannanase[5]	β-MAN	Mannan	*Asp. niger*
Gamanase[6]	GAM	Mannan	
Mix of GAL, ARA-A and ARA-B	GAL+ARA	(Arabino)galactan, arabinan	
Driselase[4]	DRI	Pectine, hemicellulose	Basidomycetes
Pectinex Ultra SP-L[6]	PEC	Pectine, hemicellulose	*Asp. aculeatus*
Rapidase Liq+[3]	LIQ	Pectine, hemicellulose	

[1] purified by Van de Vis *et al.* (1991) *(27)*

[2] purified by Rombouts *et al.* (1988) *(28)*

[3] Supplied by: DSM, Delft, The Netherlands

[4] Supplied by: Sigma, St. Louis, MO

[5] Supplied by: Megazyme, Bray, Wicklow, Ireland.

[6] Supplied by: Novozymes, Bagsvaerd, Denmark

Table 2: Monosaccharide composition (mol %) after degradation with Gammanase (GAM), Galactase + arabinofuranosidase (GAL+ARA), driselase (DRI) and pectinex Ultra SP(PEC) and dialysis.

	Fuc[1]	Rha[1]	Ara[1]	Gal[1]	Glc[1]	Man[1]	Xyl[1]	GalA[1]	GlcA[1]	Total sugars (%)[2]
Active 1	6.3	n.d	29.6	25.2	3.6	9.5	7.4	14.1	4.4	48.0
GAL+ARA	7.8	n.d	29.0	12.2	3.3	13.3	10.1	17.0	7.2	28.8
DRI	8.0	n.d	32.8	12.4	3.3	11.7	7.5	18.7	5.7	52.0
GAM	9.8	n.d	5.3	15.3	5.1	16.7	12.3	27.1	8.5	33.3
PEC	11.4	n.d	7.1	10.4	5.5	9.6	28.0	12.0	16.2	13.6

n.d. not determined

Results are averages of 2 replicates.

[1] Fuc (fucose), Rha (rhamnose), Ara (arabinose), Gal (galactose), Glc (glucose), Man (mannose), Xyl (xylose), GalA (galacturonic acid), GlcA (glucuronic acid).

[2] Sugar content (w/w %) of fraction analysed.

Table 3: Monosaccharide composition of fraction "active 3" (mol %).

	Fuc[1]	Rha[1]	Ara[1]	Gal[1]	Glc[1]	Man[1]	Xyl[1]	GalA[1]	GlcA[1]	Total sugars (%)[2]
Active 3	1.5	n.d	50.7	19.0	7.4	21.0	n.d.	0.2	0.2	56.4

n.d. not determined

Results are averages of 2 replicates.

[1] Fuc (fucose), Rha (rhamnose), Ara (arabinose), Gal (galactose), Glc (glucose), Man (mannose), Xyl (xylose), GalA (galacturonic acid), GlcA (glucuronic acid).

[2]Total sugar expressed as weight % of the total fraction.

Figure 1

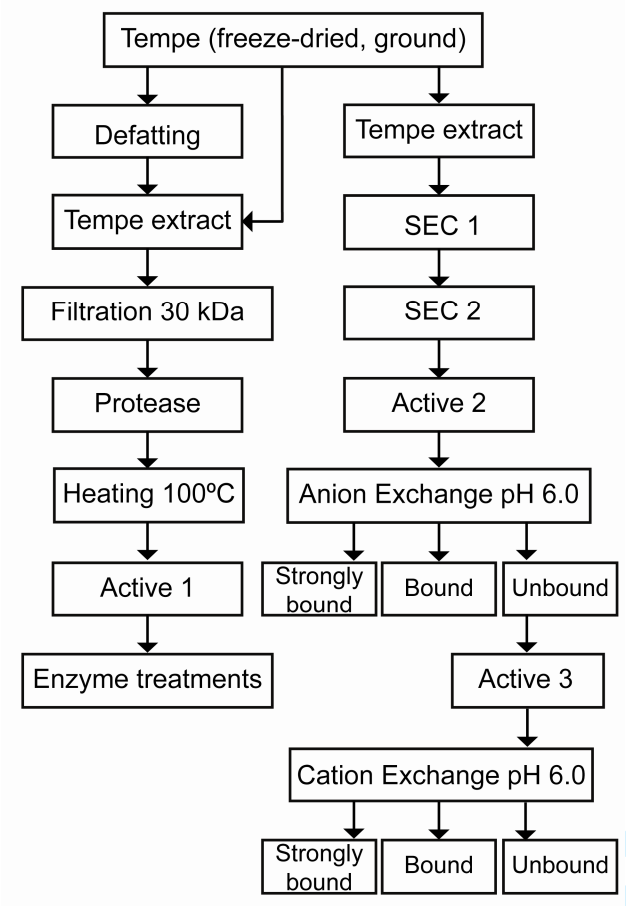

Figure 2

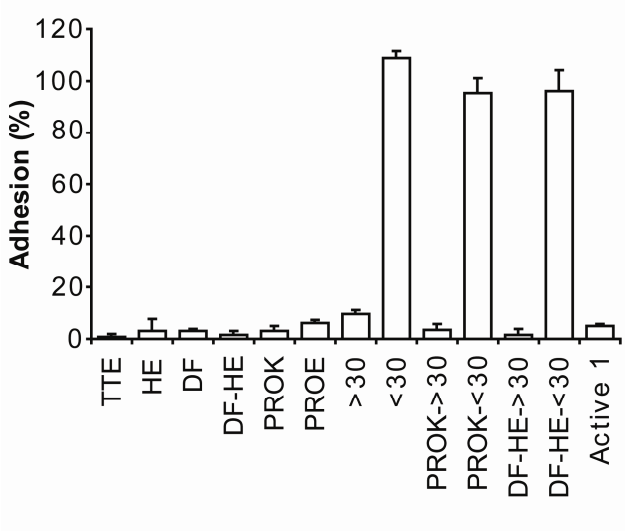

Figure 3

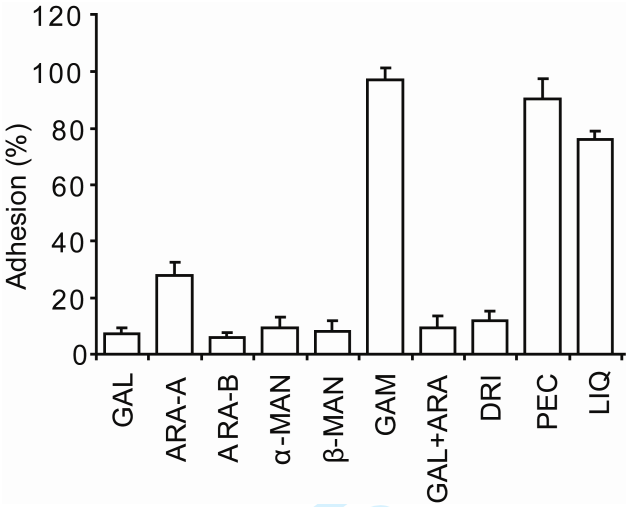

Figure 4

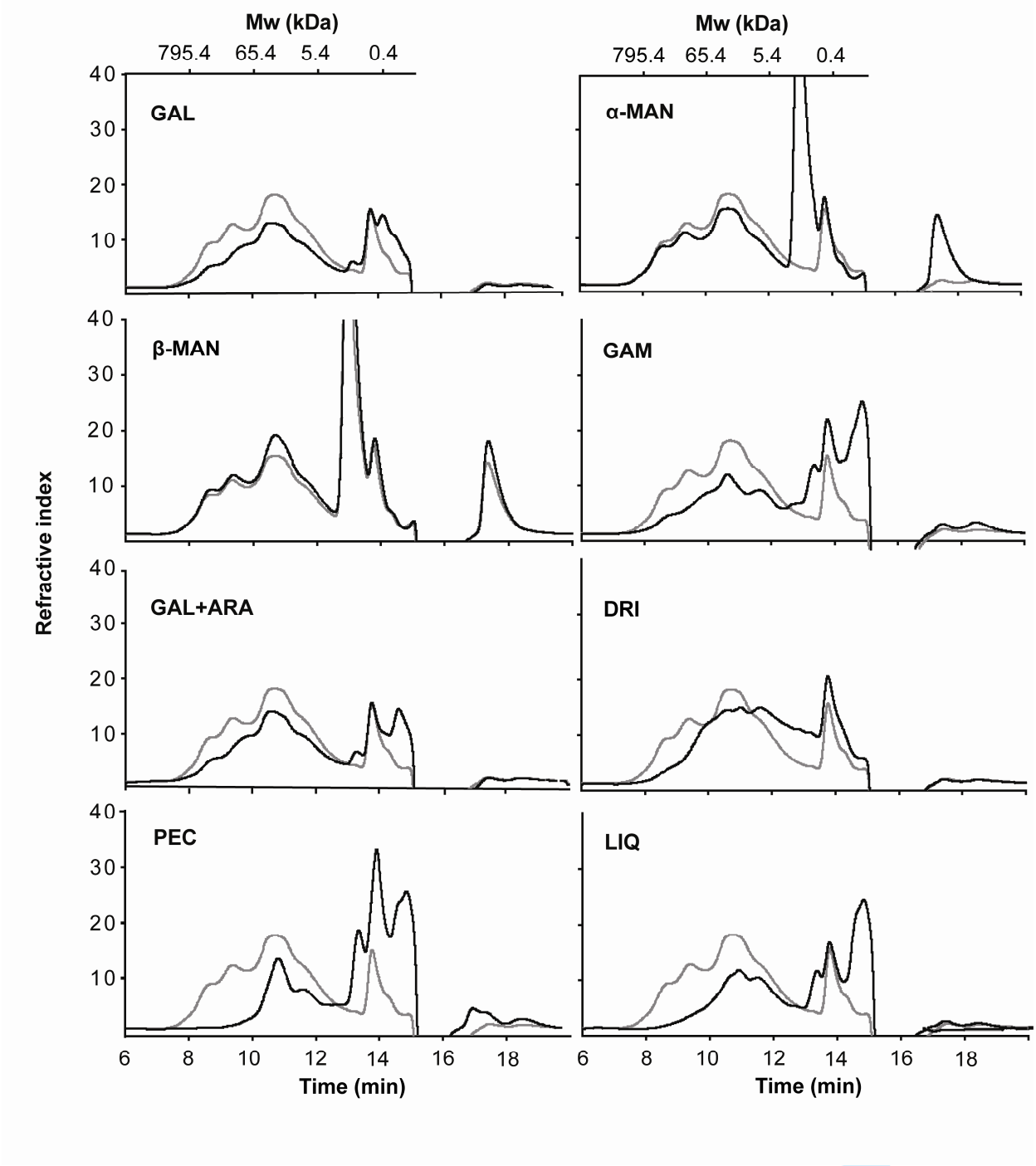
Mw (kDa)
795.4
65.4
5.4
0.4
GAL
α-MAN
β-MAN
GAM
GAL+ARA
DRI
PEC
LIQ
Refractive index
Time (min)
6
8
10
12
14
16
18
10
20
30
40

Figure 5