



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

3  
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## 15 Abstract

16 Tempe extracts can inhibit the adhesion of enterotoxigenic *Escherichia coli* (ETEC) to  
17 intestinal cells and thereby can play a role in controlling ETEC induced diarrhoea. The  
18 component responsible for this adhesion inhibition activity is still unknown. In this research  
19 we describe the purification and **partial** characterization of this bioactive component **of** tempe.  
20 After heating, defatting and protease treatment, the extracts were found to remain active.  
21 However, after treatment **with** polysaccharide degrading enzyme mixtures the bioactivity was  
22 lost. Ultrafiltration revealed the active component to be larger than 30 kDa. Further  
23 purification of the bioactive tempe extracts yielded an active fraction with an increased  
24 **carbohydrate content of higher arabinose content than the non-active fractions.**  
25 **In conclusion**, the bioactive component contains arabinose and **originates** from the arabinan or  
26 arabinogalactan side chain of the pectic cell wall polysaccharides of the soya beans. **Which is**  
27 **probably** released or formed during fermentation by enzymatic modifications.

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30

## 31 Keywords

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

### 33 **Introduction**

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

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

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

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

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

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

68 Earlier research indicated that the severity of diarrhoea in ETEC induced weaned piglets was  
69 less when these piglets were fed on tempe instead of toasted soya beans (13). Kiers et al.  
70 (2007) investigated the effect of tempe on fluid absorption in piglets and suggested that a  
71 water-soluble, high molecular mass fraction (> 5kDa) of tempe is able to protect against fluid  
72 losses induced by ETEC. As a consequence, this fraction could thus play a role in controlling  
73 ETEC induced diarrhoea (14). Furthermore, tempe extracts can inhibit the adhesion of ETEC  
74 to intestinal epithelial cells in both piglet brush border cells (15) and human epithelial (Caco-  
75 2) cells (16). Tempe extracts were found to interact with ETEC bacteria and could possibly  
76 form a layer coating (part of) the bacterial surface, which may contribute to the observed  
77 decrease of ETEC adhesion to intestinal epithelial cells (16).

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

80

## 81 **Material and methods**

### 82 **Materials**

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

### 89 **Tempe preparation**

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

105

## 106 **Bioactivity assay**

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

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

123

## 124 **Preparation of fraction “active 1”**

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

131 8.0 with 2 M NaOH every 30 min. The soluble tempe material was obtained by collection of  
132 the supernatant after centrifugation (30 min, 25000 g, 20°C). The supernatant was filtered  
133 through a folded paper filter followed by a second centrifugation step.  
134 Heat treatment was done by heating the extract for 15 min at 100°C. For protease enzyme  
135 treatment, proteinase K or pronase E were added (0.01% w/v) and incubated overnight at  
136 room temperature, followed by heating for 10 min in order to inactivate the enzymes. Then,  
137 the extracts were filtered using centrifugal filters with a membrane cut-off of 30 kDa  
138 (Centriprep YM-30, Millipore, Billerica, MA). Both filtrates and retentates were freeze-dried  
139 and used for the bioactivity assay. Also combinations of treatments were performed (see  
140 Figure 2 for combinations of treatments) and tested for bioactivity. Finally, the so called  
141 fraction “active 1” was obtained by a combination of defatting, filtration, proteinase K  
142 treatment and heating. Figure 1 shows the order of treatments to obtain fraction “active 1”.

143

#### 144 Enzyme treatments of fraction “active 1”

145 Freeze-dried fraction “active 1” ( $10 \text{ mg mL}^{-1}$ ) was solubilised in 20 mM NaOAc-buffer (pH  
146 5.0). Powdered enzymes ( $10 \text{ mg mL}^{-1}$ ) were solubilised in NaOAc buffer (pH 5.0).  
147 Carbohydrases (Table 1) were added (20  $\mu\text{L}$ ) each to 1 mL of fraction “active 1” solution and  
148 the mixture was incubated overnight at room temperature (20°C) followed by heating for 10  
149 min at 100°C.

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



156 combination with a superAW Lguard column (Tosohaas). Elution took place at 30 °C with  
157 0.2 M sodium nitrate at 0.6 mL min<sup>-1</sup>. The eluate was monitored using a refractive index (RI)  
158 detector (RI61, Shodex, New York, NY). The system was calibrated with pullulan standards  
159 (mass range 180-4.04\*10<sup>5</sup> kDa).

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

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

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

183

#### 184 **Preparation of fraction “active 2”**

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

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

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

203 Fraction “active 2” (10 mg mL<sup>-1</sup>) was dissolved in 35 mM sodium phosphate buffer (pH 6.0).  
204 Anion exchange was performed using a glass filter (diameter 25mm) filled with 3 mL of the  
205 resin (DEAE CL 6B, GE Healthcare) on top of a vacuum erlenmeyer. First, 3 mL 35 mM

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

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

220

## 221 **Results**

### 222 **Characterization of the active component in tempe**

223 Different treatments were performed to characterize the bioactive components in tempe.  
224 Figure 2 shows the bioactivity of the untreated tempe extracts as well as the extracts after  
225 heating (HE), de-fatting (DF), proteinase K and Pronase E treatment (PROK and PROE) and  
226 ultrafiltration (<30 or >30). The results show that the activity remained after de-fatting,  
227 protease treatment and heating at 100°C of the extracts. Filtering with a cut-off membrane of  
228 30 kDa showed that all activity was found in the retentate, which contained all material >30  
229 kDa. Various treatments were combined and bioactivity was present after the combination of  
230 defatting, protease treatment, heating at 100°C and ultrafiltration, and is presented as “active

231 1” in Figure 2. The results on heating and protease treatment suggest that the bioactive  
232 component is not proteinaceous.

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

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

256 not degraded to monosaccharides, but to intermediate sized molecules as shown in the  
257 chromatogram (Figure 4). After degradation with GAM and PEC the fraction “active 1” lost  
258 activity and showed a decrease in arabinose residues from 29.6 to 5.3 and 7.1 mol %,  
259 respectively, whereas galactose decreased in all enzyme-treated samples (Table 2). Despite  
260 the reduction in galactose, the GAL+ARA and DRI degraded samples remained bioactive.  
261 These two samples showed much higher molar proportions of arabinose than the GAM and  
262 PEC degraded samples. The molar proportion of galacturonic acid did not show any relation  
263 with the decreased activity caused by GAM and PEC. Also, the recovery of individual sugars  
264 after enzyme treatment was calculated. The GAM and PEC treatment resulted in a removal of  
265 88 and 93% (w/w) of all arabinose residues present in the extract before the enzyme  
266 treatment, respectively (data not shown).

267

### 268 **Purification of the active component**

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

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

283 After SEC 2, the active material eluted between 1.8 and 2.4 L (fractions 4-6) was pooled and

284 denoted as fraction "active 2". This material was eluted through an anion exchange resin  
285 column. An unbound, bound and strongly bound fraction were collected, representing 38, 13

286 and 29% (w/w) of the starting material, respectively. The bioactivity was measured and

287 fraction "active 2" showed an adhesion of  $11.1 \pm 3.6$  % (average  $\pm$  SEM) at a concentration of

288 0.1% (w/v) extract. The unbound material showed an adhesion of  $14.0 \pm 3.8$  % (average  $\pm$

289 SEM), and the bound and strongly bound fraction  $70.0 \pm 4.1$  % and  $99.3 \pm 7.5$ , respectively.

290 Obviously, the bioactive material was present in the unbound material and this part is called

291 "active 3". Subsequently, the fraction "active 3" was applied onto a cation exchange column.

292 Again, an unbound, bound and strongly bound fraction were collected, representing 14, 38

293 and 32% (w/w) of the material loaded onto the column, respectively. The bioactivity was

294 measured and the highest inhibition activity was found in the unbound material  $36.0 \pm 5.2$  %

295 (average  $\pm$  SEM) compared with the bound and strongly bound material  $84.0 \pm 11.5$  and

296  $74.0 \pm 7.2$  %, respectively, all at a concentration of 0.1% (w/v) extract. Since part of the

297 bioactivity was lost by the cationic exchange, we continued our experiments with the

298 bioactive fraction "active 3".

299 Monosaccharide analysis was performed on fraction "active 3" (Table 3). This fraction

300 contained 59 % (w/w) sugars, which is higher than found in fraction "active 1" (Table 2).

301 Also, it exhibited a higher molar proportion (50%) of arabinose than fraction "active 1". The

302 ratio between arabinose and galactose moieties had changed from 1.2:1 to 2.7:1 for active 1

303 and active 3, respectively.

## 304 Discussion

305 We examined the chemical component(s) that protect intestinal cells from adhesion of ETEC  
306 bacteria. The bioactivity was tested on piglet intestinal brush border cells, but **it** is also active  
307 in inhibiting adhesion to human cells (16). The fractions of the first size exclusion  
308 chromatographic separation (SEC 1) experiment were also tested on their inhibition capacities  
309 to human Caco-2 intestinal cells. Results showed activity in fractions 5 and 6 with an  
310 inhibition of the adhesion of ETEC K88 to the intestinal epithelial cells with 60% (no further  
311 data shown). Earlier research found an inhibition of adhesion to Caco-2 cells by total tempe  
312 extracts of almost 50%, **which may possibly reduce ETEC induced diarrhoea in humans** (16).

313 **Since our earlier research (13, 14) had shown that the protective effect of tempe against ETEC**  
314 **induced diarrhoea was not diminished by *in vitro* or *in vivo* digestion, we used non-digested**  
315 **tempe extracts in this study for reasons of convenience and to avoid purification difficulties.**

316 After ultrafiltration of the tempe extracts the bioactive component(s) was recovered in the >30  
317 kDa fraction. Also, the SEC 1 and SEC 2 experiments showed that the bioactive  
318 component(s) is intermediate in size. In the research of Kiers *et al.* (2003) the bioactive  
319 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  
320 induced fluid loss. The HPSEC patterns of these pre-digested and undigested tempe extracts  
321 were also identical in the high molecular weight part (13).

322  
323 Tempe contains a diversity of microbial enzyme activities. Proteolytic enzyme activity in the  
324 gut could degrade intestinal receptors for ETEC as was shown before for bromelain (20).  
325 After heating of the tempe extracts the proteolytic enzymes were inactivated (data not shown).  
326 Nevertheless, the tempe extracts were still bioactive after heating at 100°C (Figure 2), thus we  
327 concluded that the proteolytic enzyme activity was not responsible for the bioactivity.



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

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

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

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

351 Carbohydrates in soya beans contain mainly cell wall polysaccharides and the small sugars  
352 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

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

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

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

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

394

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398

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481

482

### 483 **Figure captions**

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

485

486 **Figure 2:** Bioactivity of tempe extracts after different treatments.

487 Concentration of the extracts is 10 mg mL<sup>-1</sup>. Bars represent average adhesion (n=12) expressed as %  
488 adhesion compared to a control without any addition of tempe extracts. Error bars represent standard  
489 error of the mean. TTE: total tempe extract, HE: cooked tempe extract, DF: defatted tempe extract,  
490 PROK: tempe extract treated with proteinase K, PROE: tempe extract treated with pronase E, >30:  
491 tempe extract >30 kDa, <30: tempe extract <30 kDa, Active 1: Combination of DF, >30, PROK and  
492 HE.

493

494 **Figure 3:** Bioactivity of fraction "active 1" after treatment with various enzymes.

495 Concentration of the extracts is 10 mg mL<sup>-1</sup>. Bars represent average adhesion (n=12) expressed as %  
496 adhesion compared to a control without any addition of tempe extracts. Error bars represent standard  
497 error of the mean. GAL: Endo- $\beta$ -1,4-galactanase, ARA-A: Arabinofuranosidase A, ARA-B:  
498 arabinofuranosidase B,  $\alpha$ -MAN:  $\alpha$ -Mannosidase,  $\beta$ -MAN: Endo- $\beta$ -mannanase, GAM: Gammanase  
499 GAL+ARA: Endo- $\beta$ -1,4-galactanase and Arabinofuranosidase A and B, DRI: Driselase, PEC: Pectinex  
500 Ultra SP-L, LIQ: Rapidase Liq+

501

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

504 GAL: Endo- $\beta$ -1,4-galactanase,  $\alpha$ -MAN:  $\alpha$ -Mannosidase,  $\beta$ -MAN: Endo- $\beta$ -mannanase ,GAM:  
505 Gammanase GAL+ARA: Endo- $\beta$ -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- $\beta$ -1,4-galactanase <sup>1,3</sup>	GAL	Galactan	<i>Asp. niger</i>
Arabinofuranosidase A <sup>2,3</sup>	ARA-A	Arabinan	<i>Asp. niger</i>
Arabinofuranosidase B <sup>2,3</sup>	ARA-B	Arabinan	<i>Asp. niger</i>
$\alpha$ -Mannosidase <sup>4</sup>	$\alpha$ -MAN	Mannan	Jack beans
Endo- $\beta$ -mannanase <sup>5</sup>	$\beta$ -MAN	Mannan	<i>Asp. niger</i>
Gamanase <sup>6</sup>	GAM	Mannan	
Mix of GAL, ARA-A and ARA-B	GAL+ARA	(Arabino)galactan, arabinan	
Driselase <sup>4</sup>	DRI	Pectine, hemicellulose	Basidiomycetes
Pectinex Ultra SP-L <sup>6</sup>	PEC	Pectine, hemicellulose	<i>Asp. aculeatus</i>
Rapidase Liq+ <sup>3</sup>	LIQ	Pectine, hemicellulose	

<sup>1</sup> purified by Van de Vis *et al.* (1991) (27)

<sup>2</sup> purified by Rombouts *et al.* (1988) (28)

<sup>3</sup> Supplied by: DSM, Delft, The Netherlands

<sup>4</sup> Supplied by: Sigma, St. Louis, MO

<sup>5</sup> Supplied by: Megazyme, Bray, Wicklow, Ireland.

<sup>6</sup> 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.

	<b>Fuc<sup>1</sup></b>	<b>Rha<sup>1</sup></b>	<b>Ara<sup>1</sup></b>	<b>Gal<sup>1</sup></b>	<b>Glc<sup>1</sup></b>	<b>Man<sup>1</sup></b>	<b>Xyl<sup>1</sup></b>	<b>GalA<sup>1</sup></b>	<b>GlcA<sup>1</sup></b>	<b>Total sugars (%)<sup>2</sup></b>
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.

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

<sup>2</sup> Sugar content (w/w %) of fraction analysed.

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

	<b>Fuc<sup>1</sup></b>	<b>Rha<sup>1</sup></b>	<b>Ara<sup>1</sup></b>	<b>Gal<sup>1</sup></b>	<b>Glc<sup>1</sup></b>	<b>Man<sup>1</sup></b>	<b>Xyl<sup>1</sup></b>	<b>GalA<sup>1</sup></b>	<b>GlcA<sup>1</sup></b>	<b>Total sugars (%)<sup>2</sup></b>
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.

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

<sup>2</sup>Total sugar expressed as weight % of the total fraction.

Figure 1

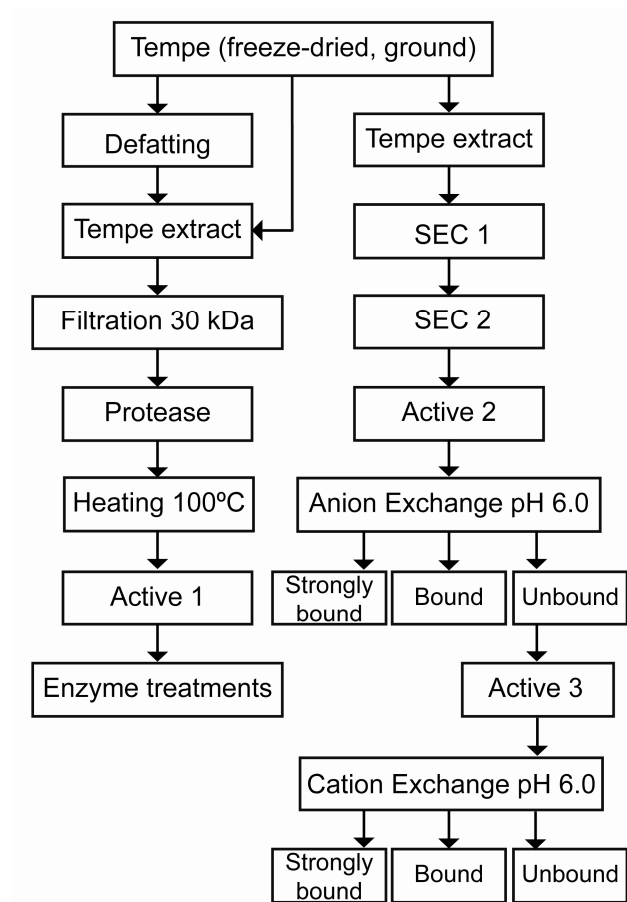
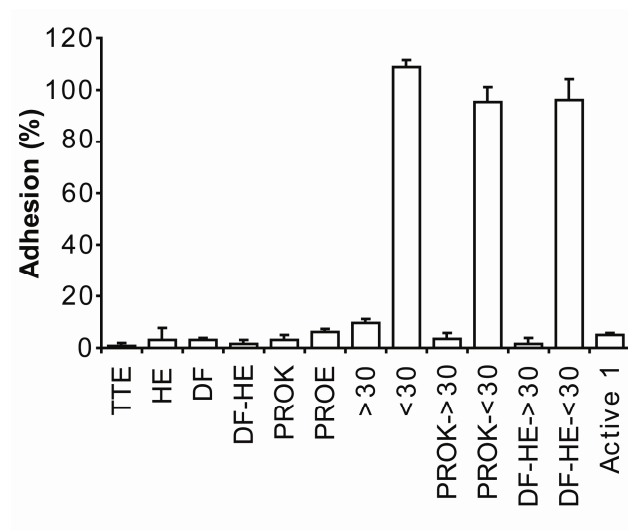


Figure 2



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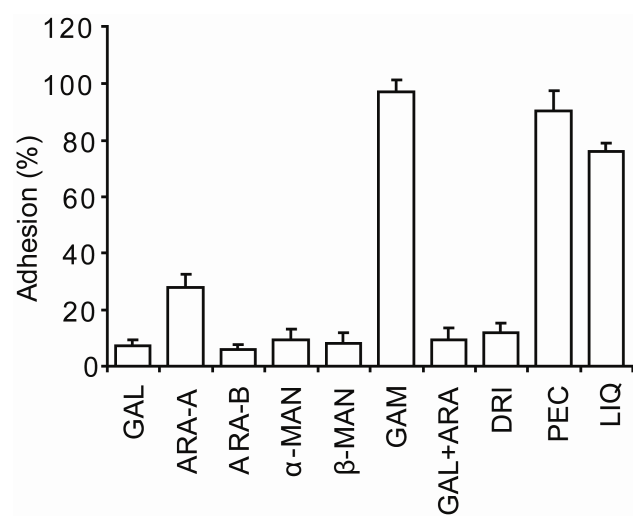
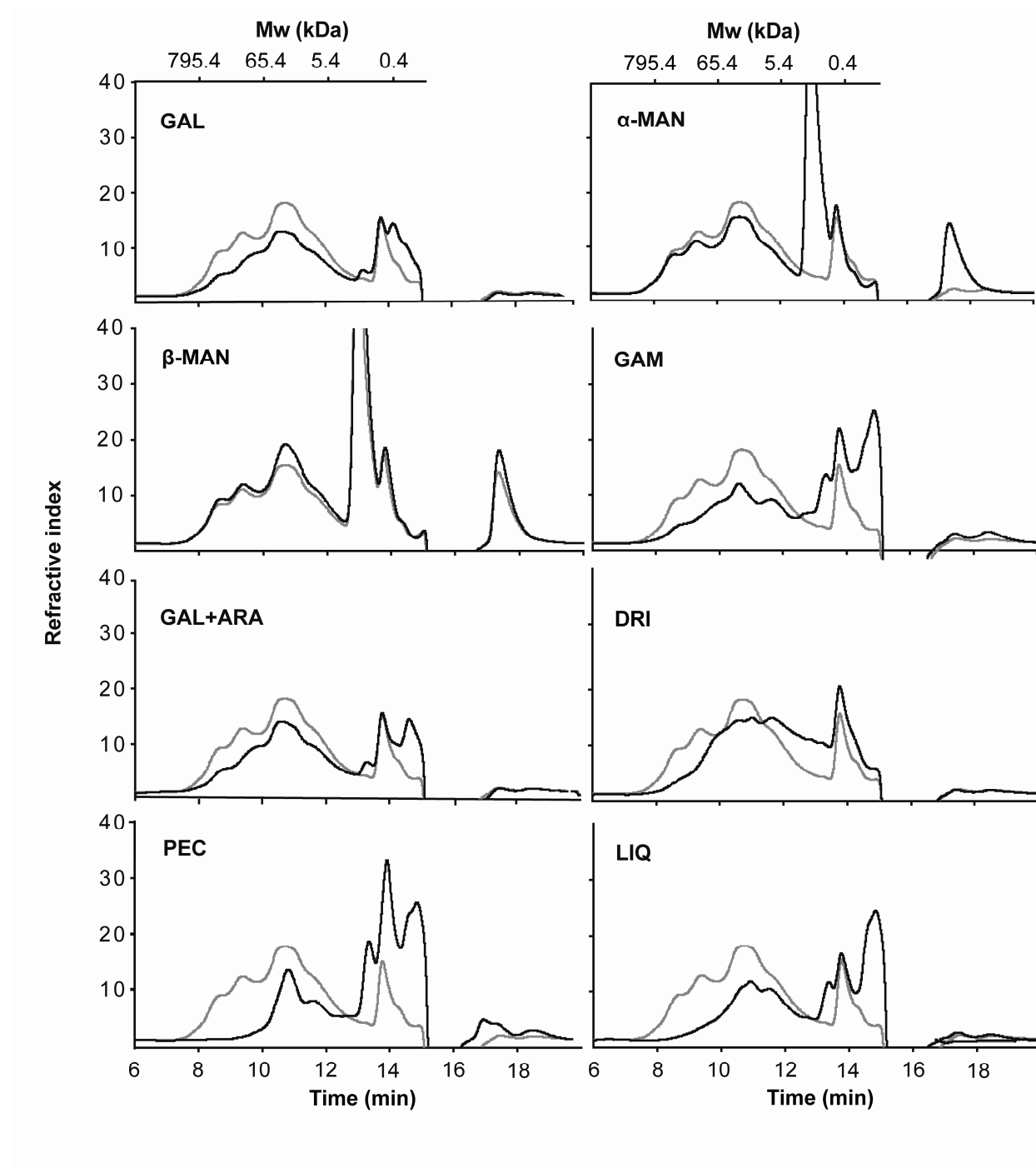
**Figure 3**

Figure 4



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Figure 5

