The effects of α-lactalbumin and glycomacropeptide on the association of CaCo-2 cells by enteropathogenic Escherichia coli, Salmonella typhimurium and Shigella flexneri

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Received 14 February 2006; revised 4 April 2006; accepted 4 April 2006.
First published online May 2006.
doi:10.1111/j.1574-6968.2006.00268.x

Editor: Peter Williams

Keywords
α-lactalbumin; glycomacropeptide; Salmonella spp.; enteropathogenic Escherichia coli; Shigella spp.; milk proteins.

Abstract
Two milk components, α-lactalbumin (α-La) and glycomacropeptide (GMP) may inhibit intestinal infection/intoxification. 3[H] thymidine-labeled enteropathogenic Escherichia coli (EPEC), Salmonella typhimurium (ATCC 6994) or Shigella flexneri (ATCC 9199) were introduced to CaCo-2 cultures and their association with CaCo-2 cells was assessed. Undigested, pepsin-digested and pepsin- and pancreatin-digested α-lactalbumin and glycomacropeptide inhibited association. Thus, milk supplemented with α-lactalbumin and glycomacropeptide might be effective in inhibiting associations of the pathogens EPEC, Salmonella typhimurium, and Shigella flexneri to intestinal cells.

Introduction
Gram-negative bacteria are important causative agents of invasive bacterial infection among neonates. Four Escherichia coli strains, enterotoxigenic (ETEC), enteraggregative (EAEC), enterohemorrhagic (EHEC) and enteropathogenic E. coli (EPEC) are of worldwide public health interest, with EPEC being a particular problem in infants (Nataro & Kaper, 1998). In addition to E. coli, rates of infection by Salmonella spp. and Shigella spp. have also been found to be significantly higher in patients with acute diarrhea compared with various other enteric pathogens (Mathew et al., 1991; Sohail & Sultana, 1998). Children, especially those less than 1 year of age are susceptible and tend to experience severe infections (Gomez & Cleary, 1998).

Evidence suggests that two human milk components, α-lactalbumin (α-La) and κ-casein-derived glycomacropeptide (GMP) may inhibit bacterial adhesion and association to the gastric mucosa (Peterson et al., 1998). Bacterial and viral association to the intestinal epithelium is a major part of the colonization process and the initial event in gastrointestinal infection. Most commonly, binding is mediated by adhesins and capsular material on the bacterial cell surface or by fimbriae or pili, which are specific for various ceramide and ganglioside glycoconjugates of the epithelial cell membranes (Neeser et al., 1995). It is therefore considered that exogenous substances containing the same carbohydrate residues such as N-acetylmuraminic acid (NeuNac) might competitively inhibit bacterial adhesion to intestinal cells and thereby inhibits colonization (Kawakami, 1997). Glycomacropeptide, containing NeuNac, has shown to prevent hemagglutination by Streptococcus mutans, Streptococcus sanguis and Actinomyces viscosus (Neeser et al., 1995). Furthermore, Strömquist et al. (1995) showed that when sections of formalin-fixed, paraffin-embedded stomach tissue were incubated with purified human κ-casein, adhesion of Helicobacter to the tissue was inhibited.

Pihlanto-Leppälä et al. (1999) demonstrated that α-lactalbumin, hydrolyzed with pepsin or trypsin, lowered the metabolic activity of E. coli JM103 to just 21% of normal after 6 h incubation. The undigested protein did not inhibit bacterial growth or metabolism. This bacteriostatic effect of the α-lactalbumin hydrolysates was found at a high concentration (25 mg mL⁻¹) as compared with a lactoferrin hydrolysate (10 μg mL⁻¹) (Saito et al., 1991). However, the study was carried out under optimum growth conditions and...
metabolism of *E. coli*, which does not necessarily represent the environment of the colon. The mechanism of α-lactalbumin hydrolysates is unclear but it is speculated that whey products affect the cytoplasmic membrane similarly to lactoferrin-derived antibacterial peptides.

In this study, it was investigated if glycomacropeptide and/or α-lactalbumin may lower rates of association of three organisms with different pathogenic mechanisms to CaCo-2 cell lines: EPEC, *Salmonella typhimurium* and *Shigella flexneri*.

**Materials and methods**

All chemicals, including purified human α-lactalbumin were obtained from Sigma (St Louis, MO) unless stated otherwise. Bovine α-lactalbumin fractions of varying purity were obtained from Arla Foods Ingredients (Viby, Denmark, Table 1).

The Radiation Use Authorization Committee at the University of California, Davis, approved all work and appropriate safety measures were taken to avoid radioactive contamination. All areas were swabbed for radioactive contamination after use.

**Enzymatic digestion of α-lactalbumin and glycomacropeptide**

Five milliliters of a 5 mg mL\(^{-1}\) solution of the proteins (Table 1) was prepared and added to 1 M HCl to lower the pH to 3.8. Pepsin was then added to give a 1 : 12.5 enzyme : protein ratio and incubated for 30 min at 37°C. Next, 1 M NaHCO\(_3\) was added to raise the pH of the solution to 7.0. A 15 mg mL\(^{-1}\) solution of pancreatin was added to 0.1 M NaHCO\(_3\) and added to the protein solution to give a 1 : 62.5 enzyme : protein ratio. The sample was incubated for 2 h at 37°C and then stored at \(-20°C\) until further use. Negative controls containing only minimal medium (MEM) were treated identically. Samples were kept for each step of the digestion process.

**Cell culture and adhesion assay**

All experiments were carried out in triplicates. CaCo-2 cells were seeded at a density of 1.0 \(\times\) 10\(^5\) mL\(^{-1}\) per well in a 24-well microtitre plate and grown 10 days past confluence at 37°C in a 5% CO\(_2\) atmosphere to develop brush border membranes. The medium was replaced every 2 days. Then, a suspension of 10\(^8\) CFU mL\(^{-1}\) of either EPEC strain E2348/69, *Salmonella typhimurium* (ATCC 6994) or *Shigella flexneri* (ATCC 9199) was seeded in 8 mL of synthetic broth (BD, Franklin Lakes, NJ) containing 50 µCi of tritium (\(^3\)H)-labeled thymidine and incubated overnight at 37°C. The next day, the CaCo-2 cells were rinsed with phosphate-buffered saline (PBS) and incubated for 30 min at 37°C in adhesion medium [9.53 g L\(^{-1}\) MEM, 7.5% (w/v) bicarbonate, 2% (v/v) FBS, 1% (w/v) D-mannose] containing 0.25, 0.05 and 0.01 mg of the proteins (undigested, pepsin digested, pepsin and pancreatin digested). Then, 10\(^6\) CFU of \(^3\)H thymidine-labeled pathogen was added to each well and incubated for 60 min at 37°C. Afterwards, the supernatant containing unattached bacteria was carefully aspirated and kept for total counts. Next, the wells were washed three times in PBS and 1 mL of 0.5% sodium dodecyl sulfate was used to remove the adherent CaCo-2 cells and attached bacteria. Finally, 10 mL of Ecolight cocktail (ICN Costa Mesa, CA) was added to the sample and shaken vigorously until thoroughly mixed with the samples. The specific activity of tritium was determined by liquid scintillation spectrometry. The relative percentage of bacteria infecting CaCo-2 cells was calculated for each pathogen by solving the relationship of the total tritium signal (supernatant + cell fraction) and the tritium signal in the cell fraction.

**Statistical analysis**

Statistical analysis was performed using the Student’s paired t-test (test protein vs. the control) for determining significance at \(P < 0.05\) unless otherwise stated. Means are given ± 1 SD. The robustness of the statistical design and analysis was confirmed at the Department of Applied Statistics, The University of Reading.

**Results**

**Specific activity of \(^3\)H labeled bacterial cultures**

Suspensions of 10\(^6\) CFU mL\(^{-1}\) of EPEC strain E2348/69, *Salmonella typhimurium* or *Shigella flexneri* had 400872, 263292 and 1489728 \(^3\)H counts per min, respectively.

**CaCo-2 cells infected with *Shigella flexneri***

The cells infected with *Shigella flexneri* (Fig. 1) and treated with the undigested test proteins had significantly lower (\(P < 0.05–0.001\)) association rates with all protein concentrations (0.25–0.01 mg mL\(^{-1}\)) than negative (untreated) control. The highest concentration had the greatest effect with rates of association increasing as the amount of

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**Table 1.** Protein composition (%) of uncharacterized α-lactalbumin fractions

<table>
<thead>
<tr>
<th>Product name</th>
<th>α-la (H)</th>
<th>α-la68</th>
<th>α-la25</th>
<th>GMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-lactalbumin</td>
<td>90</td>
<td>68</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>β-lactalbumin</td>
<td>NT</td>
<td>22</td>
<td>NT</td>
<td>10</td>
</tr>
<tr>
<td>GMP</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>Other</td>
<td>10</td>
<td>10</td>
<td>65</td>
<td>8</td>
</tr>
</tbody>
</table>

α-la (H), human α-lactalbumin (purity: 90%; Sigma); α-la68, bovine α-lactalbumin (purity: 68%; Arla Foods); α-la25, semi-pure α-lactalbumin (purity: 25%; Arla Foods); GMP, κ-casein glycomacropeptide (purity: 80%; Arla Foods); NT, not tested.
protein decreased. Similar results were observed for the cells treated with pepsin-digested proteins. Glycomacropeptide concentrations lower than 0.05 mg mL\(^{-1}\) no longer had a significant inhibitory effect of association. For cells treated with pepsin- and pancreatin-digested proteins, association rates with \textit{Shigella flexneri} were significantly lower for protein concentrations between 0.25 and 0.05 mg mL\(^{-1}\). Concentrations lower than 0.05 mg mL\(^{-1}\) no longer had a significant inhibitory effect of association.

\textbf{CaCo-2 cells infected with EPEC}

For cells infected with EPEC (Fig. 2), the opposite effect was observed. Undigested test proteins had no significant effect on association rates with the exception of purified human \(\alpha\)-lactalbumin and glycomacropeptide, which both had a significant effect (\(P < 0.01\)) at 0.25 mg mL\(^{-1}\). Proteins digested with pepsin had a significant effect (\(P < 0.05–0.001\)) on associations rates with an observed decrease of inhibition at lower protein concentrations. All test proteins digested with pepsin and pancreatin significantly lowered rates of association (\(P < 0.05–0.001\)).

\textbf{CaCo-2 cells infected with \textit{Salmonella typhimurium}}

For cells infected with \textit{Salmonella typhimurium} (Fig. 3) the test proteins had a varying effect. Association was significantly lower (\(P < 0.05\)) at protein concentrations ranging from 0.25 to 0.05 mg mL\(^{-1}\) compared with the negative control for undigested and pepsin-digested proteins. Association rates generally ranged between 5% and 10% compared with 15% for the negative control. At lower concentrations, the proteins did not have any effect. Finally, a significantly greater effect (\(P < 0.05\)) was observed for pepsin- and pancreatin-digested proteins compared with the other the protein fractions. Association was significantly reduced (\(P < 0.01\)) compared with the negative control.
Diarrhoea-causing gastrointestinal infections are a major cause of infant morbidity and mortality in both the developing and industrial world. EPEC is among the commonest bacterial causes (Cravioto et al., 1988) along with Salmonella spp. (Mathew et al., 1991) and Shigella spp. (Sohail & Sultana, 1998). The influence of α-lactalbumin and glycomacropeptide on the association of these pathogens to the human intestinal mucosa was assessed in this study. While all three pathogens where inhibited by both α-lactalbumin and glycomacropeptide, their rate of association with CaCo-2 cells varied depending on mode of treatment digestion. Salmonella typhimurium was most inhibited in wells containing pepsin- and pancreatin-digested α-lactalbumin and glycomacropeptide, whereas EPEC was most inhibited in wells containing pepsin and pancreatin as well as just pepsin-digested α-lactalbumin and glycomacropeptide. On the other hand, Shigella flexneri was most inhibited in wells containing undigested or pepsin digested α-lactalbumin and glycomacropeptide added. This would lead to the conclusion that the fragments produced by pancreatin and pepsin digestion of α-lactalbumin and glycomacropeptide were most active for Salmonella typhimurium, while peptide obtained from pepsin digestion where most active against EPEC. Undigested compounds had most effect on Shigella flexneri even though overall activity against the pathogen was less than for the other two organisms. The cause of this could be the different pathogenic mechanisms employed by the organisms studied. EPEC bind to the epithelial cells by a well-described mechanism to cause infection, giving more access for α-lactalbumin and glycomacropeptide to inhibit or stop the infection process. This would confirm previous studies showing that α-lactalbumin, hydrolyzed with pepsin, lowered the metabolic activity of E. coli JM103 to just 21% of normal after 6 h incubation. The undigested protein did not inhibit bacterial growth or metabolism (Pihlanto-Leppälä et al., 1999).

Discussion

A similar mechanism of action for α-lactalbumin could be the cause of Salmonella inhibition by pepsin- and pancreatin-digested α-lactalbumin. While Salmonella are much more likely to be intracellular pathogens, cells first need to undergo ‘ruffling’ (deformation of the cell membrane) and extensive actin rearrangements in the vicinity of the invading bacteria before the bacteria are engulfed. Pelligrini et al. (1999) found that proteolytic digestion of α-lactalbumin by trypsin and chymotrypsin yielded three peptide fragments with bactericidal properties against gram-positive bacteria and a limited function against gram-negative organisms. Two of the fragments, a pentapeptide with sequence EQLTK (residues 1–5) and GYGGVSL-PEWVCTTF-ALCEK (residues 17–31) were obtained by trypsin digestion. Chymotrypsin digestion yielded the peptide CKDDQNPH-ISCDKF (residues 61–68) which would cause bacterial adhesion to the sialic acid group on the cell before invasion but also produce exotoxin which causes severe inflammation and bloody diarrhea which could interfere with the action of α-lactalbumin hydrolysates. The rapid multiplication of Shigella could be a cause for the reduced effectiveness of both α-lactalbumin and glycomacropeptide as bacterial populations would recover too fast for α-lactalbumin to have an effect.

Glycomacropeptide inhibited the association of the EPEC and Salmonella with CaCo-2 cells by a different but equally effective mechanism based on its sialic acid substructure which would survive the digestion steps used in this study largely intact. The cause for this would be a decoy effect which would cause bacterial adhesion to the sialic acid substructure of glycomacropeptide instead of the intestinal
epithelium. Previous studies showed glycomacropeptide preventing hemagglutination by *Streptococcus mutans*, *Streptococcus sanguis* and *A. viscosus* (Kawakami, 1997). In addition, Strömquist et al. (1995) confirmed that when sections of formalin-fixed, paraffin-embedded stomach tissue were incubated with purified human κ-casein, adhesion of bacteria to the tissue was inhibited.

In summary, α-lactalbumin and its hydrolysates in addition to glycomacropeptide were shown to significantly lower the association and internalization compared with an untreated control. While it is possible that glycomacropeptide is active through a decoy effect, the mechanism by which α-lactalbumin exerts its activity remains unclear. It is possible that the whey product disturb cellular metabolism by increasing cell permeability causing bacteria to consume more ATP in order to maintain normal membrane integrity and functions. Therefore, it is possible that the bacteria were cleared from the cells before association and infection could take place. As a whole, peptide fractions containing α-lactalbumin and glycomacropeptide inhibit the association of the pathogens EPEC, *Salmonella typhimurium*, and *Shigella flexneri* with intestinal cells and may thus prevent infection. Therefore, milk supplementation with α-lactalbumin and glycomacropeptide might be effective in preventing invasive bacterial gastrointestinal infections among neonates.

### Acknowledgements

Funded through PhD studentships by Arla Foods Ingredients amba (Arla Foods Ingredients amba, Viby, Denmark).

### References


