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Bacterial recognition of thermal glycation products derived from porcine serum albumin with lactose

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Recently, glyco-therapy is proposed to prevent the interaction of bacterial lectins with host ligands (glycoconjugates). This interaction represents the first step in infection. Neoglycans referred to as PSA-Lac (PSA-Glu (β1–4) Gal) were obtained by conjugation of porcine serum albumin (PSA) with lactose at 80°C, 100°C and 120°C. Characterization studies of the products showed that PSA could contain 1, 38 or 41 added lactoses, depending on the reaction temperature. These neoglycans were approximately 10 times more glycated than PSA-Lac obtained in previous work. Lactose conjugation occurred only at lysines and PSA-Lac contained terminal galactoses as confirmed by *Ricinus communis* lectin recognition. Furthermore, *Escherichia coli* K88+, K88ab, K88ac and K88ad adhesins showed affinity toward all PSA-Lac neoglycans, and the most effective was the PSA-Lac obtained after 100°C treatment. *In vitro*, this neoglycan partially inhibited the adhesion of *E. coli* K88+ to piglet mucin (its natural ligand). These results provide support for the hypothesis that glycated proteins can be used as an alternative for bioactive compounds for disease prevention.

INTRODUCTION

Oligosaccharide chains of glycoconjugates on eukaryotic and prokaryotic cell surfaces form the first line of interaction with the extracellular world through their recognition by carbohydrate-binding proteins (Varki, 1993). These interactions mediate the adhesion of many pathogenic bacteria to the host cell as the initial step of infectious disease (Sharon & Lis, 1989). Blocking of these interactions at an early stage following the exposure of the host to pathogens could prevent or reduce the infection (Sharon, 2006). One of the major drawbacks to the anti-adhesion therapy is the lack of appropriate glycoconjugates. Thus, the synthesis of glycoprotein moieties that mimic glycoconjugates, also referred to as neoglycoconjugates, represents an alternative strategy to produce single glycoforms for anti-adhesion use (Wong, 1995). The development of a simple and low cost process for neoglycoconjugate synthesis by chemical means has been viewed with skepticism because of the complexity of glycan molecules. Nevertheless, previous work has shown that glycation based on the Maillard reaction can serve as the basis for the synthesis of neoglycans as tools that can be used for biological recognition (Stowell & Lee, 1978; Boratynski & Roy, 1998).

The early stage chemistry of non-enzymatic protein glycation involves an initial nucleophilic substitution reaction between sugar carbonyl groups and amino groups of proteins to form a Schiff´s base that undergoes spontaneous changes known as the Amadori rearrangement (Singh *et al.*, 2001). Controlled and limited glycation at this point enables a direct attachment to protein without any prior modification or chemical activation of the reagents (Ledesma-Osuna *et al.*, 2008). Sarabia-Sainz *et al.* (2009) and Ledesma-Osuna *et al.* (2008) demonstrated that lactosylated porcine serum albumin (PSA) neoglycans obtained by Maillard reaction interact with galactose-specific K88 adhesin. This microbial lectin expressed on the *Escherichia coli* K88 cell surface is responsible for the initial infection of piglets that results in diarrhea. A definition of the reaction conditions of glycation, such as temperature, water activity and time of reaction, is necessary for neoglycan synthesis optimization in order to obtain neoglycans that can be used to prevent or deter *E. coli* K88 adhesion to piglet intestine cell surfaces, and thereby reduce infection. Here the effect of temperature in PSA glycation with lactose was studied.

MATERIALS AND METHODS

Materials. PSA was purchased from Pel-Preez Biologicals (Arkansas, USA). Biotin-labeled *Ricinus communis* lectin was from Vector (Burlingame, CA, USA) and BSA-Gal α (1–3) Gal was acquired from Glycorex (Sweden). All other chemicals were analytical grade from Sigma-Aldrich (St Louis, MO, USA).

Preparation of glycated proteins. Glycation of PSA was conducted according to Kańska and Boratyński (2002) with minor modifications. Briefly, 80 mg of PSA (in 10 mM phosphate buffer, pH 6.5) was mixed with 80 mg of D-lactose to attain 1:200 molar ratio. Samples

were frozen at −40°C and freeze-dried (Virtis Benchop 6.6, NY, USA). Portions of the resulting powder (each of 20 mg) were incubated at 80°C, 100°C and 120°C for 30 min. Heat treatments were interrupted by the addition of cold double-distilled water. Free lactose was removed by extensive dialysis in double-distilled water at 4°C. Finally, samples were freeze-dried and stored (in sealed containers) at −20°C until further analysis.

Polyacrylamide gel electrophoresis. PSA and PSA-Lac neoglycans were analyzed under denaturing and reducing conditions using 10% polyacrylamide gels (SDS/PAGE) according to Laemmli (1970). Samples containing 10 μg of proteins were loaded to gels, and stained with 1% Coomassie blue in 30% methanol and 10% acetic acid.

Spectrometry analysis. PSA and PSA-Lac neoglycans were sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona, USA) for molecular mass determination by MALDI-ToF. Mass spectra were acquired using an Applied Biosystems Voyager DE-STR (Framinham, MA, USA), operating a 337 nm nitrogen laser. Samples were resuspended in a solution containing 0.1% of trifluoroacetic acid (TFA) to give final concentration of 2 μg/μL. Samples (5 μL) were mixed with 5 μL of a saturated α-cyano-4-hydroxy-cinnamic acid solution in 50% acetonitrile/50% water containing 0.1% of TFA, and 1 μL was spotted on the target plate and allowed to air-dry prior to mass analysis. Mass spectra were collected in linear mode with an accelerating voltage of 25 000 V.

Enzymatic digestion of neoglycans. For identification of carbohydrate-modified amino acids, untreated PSA and PSA-Lac 100°C were digested with 1 mg/mL solution of trypsin (Shevchenko et al., 1996). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was performed on the protease-treated samples. One microgram of digested protein was injected onto a linear quadrupole ion trap ThermoFinnigan LTQ mass spectrometer (San Jose, CA, USA) equipped with a Michrom Paradigm MS4 HPLC, a SpectraSystems AS3000 autosampler, and a nanoelectrospray source. Peptides eluted from a 15 cm pulled tip capillary column using a gradient of 0–65% solvent B (98% methanol/ 2% water/0.5% formic acid /0.01% trifluoroacetic acid) over a 60 min period at a flow rate of 350 mL/min. Mobil phase (solvent A) included 0.5% formic acid and 0.01% trifluoroacetic acid in water. The LTQ electrospray positive mode spray voltage was set at 1.6 kV, and the capillary temperature at 180°C. Data scanning was performed by the Xcalibur v1.4 software (Andon et al., 2002). Lactose-modified peptides were identified by comparison with peptides of unmodified PSA.

Enzyme linked lectin recognition assays (ELLA assays). Recognition of PSA-Lac by biotin-labeled RCA1 lectin (specific for galactose) was done by Enzyme-linked lectin assay (ELLA) according to Sarabia-Sainz et al. (2009). Briefly, polystyrene ELISA microtitre plates were coated with 100 μL of diluted solutions of PSA-Lac treatments in 50 mM sodium bicarbonate buffer (pH 9.5). PSA-Lac solutions were serially diluted (from 25 to 1 ng), applied to ELISA plate wells and incubated for 16 h at 4°C. Following adsorption, wells were blocked for 3 h with 20 mM phosphate buffered saline containing, 0.05% Tween 20, pH 7.5 (TPBS) and 1.5% BSA. Aliquots (100 μL) of biotinylated RCA1 lectin (5 μg/mL) in PBS, pH 7.5, were added and incubated for 2 h. After washing with TPBS, the plates were

incubated with avidin-peroxidase (1:1 000, v/v, in PBS, pH 7.5) for 1 h, and o-phenylendiamine was used to reveal lectin binding. Color development was stopped after 10 min by adding 100 μL of 0.5 M H_2SO_4 and solution absorbance was measured using an ELISA 680 microplate reader (BioRad, CA, USA) at 450 nm.

Piglet mucin isolation. Duodenum sections from nine healthy piglets (17 days old) were obtained and mucins released according to Sarabia-Sainz et al. (2009).

E. coli K88+ growth. E. coli K88+ strains isolated from pigs, kindly donated by Dr. Carlos Eslava from Universidad Nacional Autonoma de Mexico, and E. coli subtypes (K88ab, K88ac and K88ad) from American Type Culture Collection (ATCC) were maintained as frozen stocks. Overnight cultures grown in Nutrient Broth (DIFCO), using a shaker at 37°C, were harvested by centrifugation. Bacteria pellets were washed twice in PBS, pH 7.5 and suspensions adjusted to 0.4 units of optical density at 600 nm (about 2×10^8 cfu mL−1) before use.

E. coli K88 adhesion assays (ELLA). ELLA assays were conducted according to Yacoby et al. (2007) with minor modification. Briefly, 100 μL of E. coli K88+ suspensions (2×10^8 cfu mL−1) were applied to ELISA plate wells and fixed with 1% glutaraldehyde (in PBS, pH 7.5) for 2 h. After three TPBS washes, nonspecific interactions were blocked for 1 h with TPBS containing 1.5% BSA, and further incubated for 2 h with 100 μL of biotin-labeled PSA-Lac (10 μg/mL) heat-treated or not. Following two TPBS washes, the plates were incubated with streptavidin-peroxidase (1:1000) for 1 h and o-phenylendiamine was used to detect bacterial adhesion. Color development was stopped after 10 min by adding 100 μL of 0.5 M H_2SO_4. Absorbance was measured as described before (Sarabia-Sainz et al., 2009). ELLA assays were also conducted with the different E. coli K88 variants (K88ab, K88ad, and K88ad) and PSA-Lac glycated at 100°C.

Inhibition of E. coli K88+ adhesion to piglet mucins. To test for inhibition of adhesion of E. coli K88+ to piglet mucins by PSA-Lac, 50 μg of intestinal piglet mucin was immobilized at concentrations of 50 μg in ELISA plates for 16 h at 4°C. Subsequently, biotin-labeled bacteria (5×10^8 cfu mL−1) that was pre-incubated for 1 h with PSA-Lac-100°C (3 μg/μL) at room temperature was overlaid on the ELISA plate wells and detection proceeded as described for the lectin assays.

RESULTS AND DISCUSSION

Evaluation of PSA glycation

SDS/PAGE under reducing conditions was used to evaluate the extent of PSA glycation (Fig. 1A). PSA heated in the presence of lactose showed a temperature-dependent reduction of migration (mass increment). PSA-Lac 80°C showed (Fig. 1A, lane 2) slight reduction of migration when compared to native PSA (Fig. 1A, lane 1). Glycation at 100°C or 120°C are products with a greater mass (Fig. 1A, lanes 3 and 4).

The PSA-Lac products glycated at 100°C presented two electrophoretic bands with different mobility and staining intensity: a darker band with an approx. 10% increase in apparent molecular mass and a faint band that corresponded to a mass increment increase of about 18% (Fig. 1A, lane 3). These results suggested that glycation under these conditions led to the formation of products with different number of lactose.

Mass determination

Mass spectroscopy has been used to assess protein molecular masses and is one of the best methods to estimate mass changes such as those resulting from protein modifications (Kislinger *et al.*, 2002; Sun *et al.*, 2005). MALDI-ToF MS was used to evaluate the molecular mass of PSA glycated at different temperatures. The spectra of untreated and glycated PSA products are shown in Fig. 1B–E. Their comparison reveals clear differences. The most abundant molecular ion of native PSA was 66 384.01 m/z (Fig. 1B). PSA-Lac glycated at 60 °C showed the same spectra as that of native PSA (not shown) indicating no lactose addition to PSA at this temperature. However, under all other temperature conditions, an increased PSA molecular mass was observed. The most abundant ion of PSA-Lac glycated at 80 °C (Fig. 1C) had an m/z of 66 785.49. PSA-Lac produced by incubation at 100 °C (Fig. 1D) yielded two main ions. Peak maximum was found at 66 422.80 m/z, and a broad and heterogeneous peak with approximate maximum at 78 323.85 m/z was also present. The resulting mass differences of PSA-Lac formed at 100 °C are probably results of multiple modifications with lactose, primarily reflecting the heterogeneity of Maillard reaction (Chevalier *et al.*, 2002) and are in agreement with the electrophoretic patterns data (Fig. 1A, lane 5). The spectrum of PSA-Lac glycated at 120 °C also showed a heterogeneous peak with a maximum of 79 790.64 m/z (Fig. 1E). MALDI-ToF MS data confirmed the electrophoretic analysis
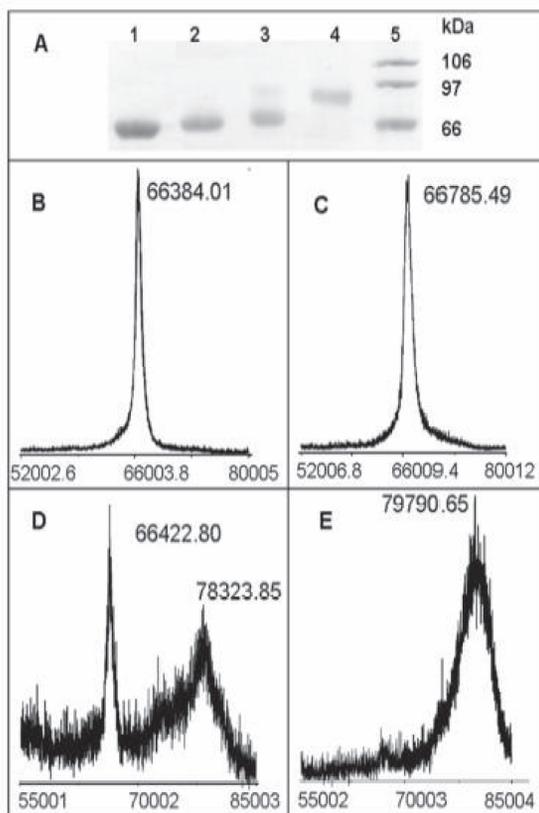
and demonstrated that the higher the temperature of reaction the greater the product mass.

A summary of the molecular masses of the most abundant ions in the treatment of PSA-Lac and the number of lactoses added by each treatment is shown in Table 1. PSA-Lac heated at 80 °C for 30 min presented addition of one lactose, while the glycation treatments at the higher temperatures (100 °C and 120 °C) were more effective, adding 38 and 41 molecules of lactose to PSA, respectively. The level of PSA glycation was higher for this study than for our previous work where four lactose molecules were added to PSA-Lac (Sarabia-Sainz *et al.*, 2009) using the method described by Fenaille *et al.* (2002). Fenaille *et al.* (2002) used solid-state synthesis of neoglycans that involved protein glycation under conditions of controlled relative humidity (RH) at 25 °C for 7 days, followed by heat treatment for different periods of time. Thus, the modified method of Kańska and Boratyński (2002) used in this study is less time consuming and more efficient for the synthesis of PSA-Lac neoglycans. PSA-Lac 100 °C was chosen for subsequent work.

Sites of glycation

In proteins, the N-terminal amino acid, lysines (Lys) and arginines (Arg) are the most frequently glycated amino acids. PSA primary sequence has 58 Lys and 33 Arg residues that are available for carbohydrate conjugation. However, Sarabia-Sainz *et al.* (2009) have shown that Lys is the most frequently modified amino acid. To determine sites of glycation, PSA-Lac (100 °C) was treated with trypsin, and the peptides and glycopeptides were identified and sequenced by mass spectrometry. Figure 2 shows the sequence of peptides where Lys residue(s) were glycated. Under these conditions, neither Arg nor the N-terminal amino acids were modified. PSA-Lac glycated at 100 °C contained 37 Lys residues modified with lactose (indicated in bold), while 21 remained intact. Further data analysis showed that in PSA-Lac twenty Lys that had neighboring hydrophobic residues and eight pairs of adjacent Lys were glycated. The accessibility of amino-acid residues appears to play an important role in protein glycation (Nacharaju & Acharya, 1992). The site selectivity of glycation in proteins is suggested to be a consequence of the three dimensional structure that determines the availability of amino acids to bind carbohydrates (Nacharaju & Acharya, 1992), as well as the ability of a given site to undergo the Amadori rearrangement (Acharya *et al.*, 1983). The latter capacity, in turn, depends on molecular microenvironmental conditions. Other factors have also been shown to be important including the facility for Amadori rearrangement formation at the glycation sites (Shapiro *et al.*, 1980), and the nature

Figure 1. Mass spectra by MALDI ToF of PSA-Lac obtained at various temperatures
(A) SDS/PAGE of PSA treated at various temperatures. Lane 1, PSA untreated; Lanes 2, 3, 4, PSA glycated with lactose at 80 °C, 100 °C and 120 °C, respectively; Lane 5, molecular mass standards. **(B)** MALDI ToF mass spectra for PSA untreated; **(C)** PSA-Lac 80 °C; **(D)** PSA-Lac 100 °C; **(E)** PSA–Lac 120 °C.

Table 1. Molecular mass of most abundant ions for PSA-Lac and numbers of lactose units added on glycated PSA

Treatment	Molecular mass[a] (Da)	Lactoses added[b]
PSA untreated	66 384.01	–
PSA-Lac 80 °C	66 785.49	1
PSA-Lac 100 °C	66 422.80 and 78 323.85	0 and 38
PSA-Lac 120 °C	79 790.65	41

[a]Mass of most abundant ion. [b]To determine the number of carbohydrate molecules conjugated, mass difference (glycated–untreated PSA) was divided by 324 Da (lactose residue mass).
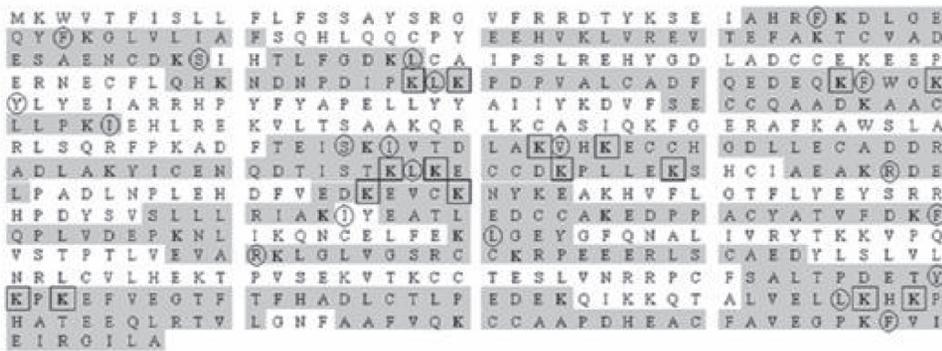
Figure 2. Peptide mapping by mass spectroscopy of lysine residues modified in PSA-Lac 100 °C.
Peptide sequences were determined from trypsin digestion of PSA-Lac 100 °C. K, Lysines modified with lactose. O, Vicinal hydrophobic amino acids. O□ = Pairs of glycated K.

of amino-acids surrounding the Lys residues (Baynes *et al.*, 1989). The most reactive Lys residues appear to be near hydrophobic amino acids in the primary structure (Mennella *et al.*, 2006). Further, the proximity of another Lys in the primary structure has been reported to promote human serum albumin glycation (Garlick & Mazer, 1983). In this study, the addition of multiple lactoses to PSA-Lac at 100 °C probably was influenced by the reaction temperature (Kańska & Boratyński, 2002).

Recognition of PSA-Lac by soluble lectin assay (ELLA assays)

Except for monosaccharides, the conjugation of carbohydrates to protein makes them susceptible to biological recognition by plant lectins (Blome & Schengrund, 2008; Ledesma-Osuna *et al.*, 2008). ELLA assays were used to confirm that galactose residues were available from the produced neoglycans. PSA-Lac obtained at 100 °C and 120 °C were strongly recognized by RCA1 lectin (specific for β-galactose), while glycation products produced at 80 °C presented similar interaction as

Figure 3. (A) ELLA assay for the recognition of PSA-Lac by *Ricinus communis* lectin
Protein (3 μg) was adsorbed in ELISA plate wells, then overlaid with biotinylated RCA lectin. **(B)** ELLA assay for interaction of *E. coli* K88+ and PSA-Lac. Bacteria was adjusted to 0.5 units of absorbance (660 nm) and 100 μL were adsorbed in ELISA plate wells, then overlaid with biotinylated piglet mucin, biotinylated untreated PSA or biotinylated PSA-Lac.

did untreated PSA (Fig. 3A). PSA glycated at 80 °C has only one carbohydrate ligand that appeared unavailable to interact with the lectin. Meanwhile, PSA-Lac 120 °C showed higher interaction than BSA-Gal α(1–3) Gal (positive control). This commercial neoglycan has 10–15 spacer arms with terminal galactoses that are recognized by RCA (Sarabia-Sainz *et al.*, 2009). PSA-Lac 100 °C (38 lactoses) matched the degree of interaction between the lectin and BSA-Gal α(1–3) Gal. This observation also suggests that not all galactoses have the same exposure.

Glycation with lactose (galactose-β(1–4)-glucose) produced covalent binding of the reducing sugar (glucose) with the amino group side chain of Lys residues of PSA. The product makes galactose available for recognition. In this context, this study data substantiate that the method used here for the glycation of PSA did not alter the structure of galactose, and further, confirms that optimal lactose addition to albumin occurs at higher temperatures. In contrast, glycation by glucose can occur at lower temperatures, as is the case for proteins in the blood of diabetic patients; however, this modification is not recognized by plant lectins (Ledesma-Osuna *et al.*, 2008).

Bacterial adhesin recognition

The interaction between *E. coli* K88+ adhesin and PSA-Lac neoglycans was established using a modified ELLA assay. Bacteria was immobilized in ELISA plates using glutaraldehyde, subsequently adhesin interaction with neoglycans was evaluated (Fig. 3B). Positive control included piglet intestine mucin, the natural ligand for K88+ adhesin, and untreated PSA (negative control). All PSA-Lac treatments were recognized by bacterial adhesins and PSA-Lac 100 °C showed the strongest signal. *E. coli* adhesins also interacted with PSA-Lac 80 °C and PSA-Lac 120 °C, as well as with mucin. PSA showed a slight association, probably due to nonspecific binding activity (He *et al.*, 2006).

As opposed to ELLAs with RCA1 lectin where PSA glycated at 120 °C was more readily recognized than that prepared at 100 °C, in adhesin assays, PSA-Lac 100 °C interaction was greater. This behavior could be attributed to albumin aggregation at the higher glycation temperature (120 °C), that interfered with the bacterial adhesion. Alternatively with the soluble lectin, its size likely also facilitates or hinders interaction with the ligand (Sharon & Lis, 1989).

The antigenic serological variants of K88 adhesin (K88ab, K88ac and K88ad) result in a small number of
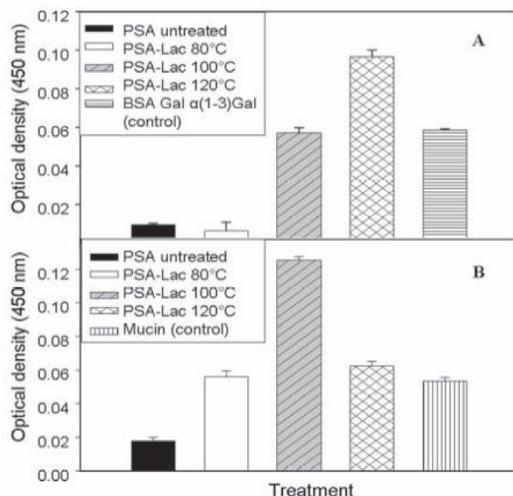
Figure 4. ELLA assay for interaction of PSA-Lac 100°C in the *E. coli* adhesins variants
E. coli variants (K88+, K88ab, K88ac, K88 ad) were adjusted at 0.5 units of optical density (660 nm) and 100 µL were adsorbed in ELISA plate wells; then overlaid with biotinylated piglet mucin, biotinylated untreated PSA or biotinylated PSA-Lac.
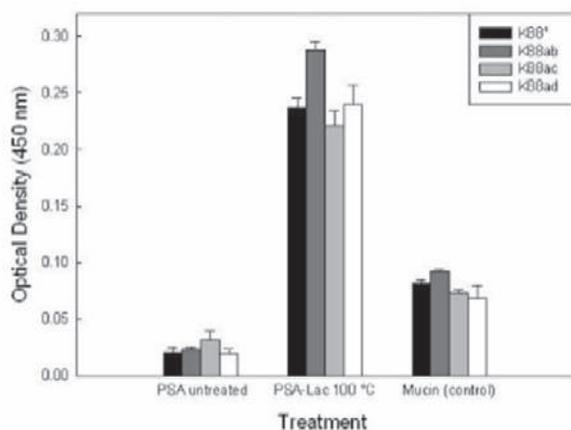
amino acid substitutions that conferred on them a different, but related, carbohydrate-binding specificity (Willemse & Graaf, 1992; Verdonck *et al.*, 2004). Grange *et al.* (2002) showed that all variants of K88 recognize structures ending in galactose β links, such as in lactose. PSA products were used to test for *E. coli* adhesion variants' interactions. Figure 4 shows that PSA-Lac 100°C was recognized by all three *E. coli* adhesin variants. However interaction was greater with K88ab. The adhesins also interacted with piglet mucin but with lower affinity. Thus, it is noteworthy that PSA-Lac 100°C presented a higher affinity for *E. coli* K88 than did mucin, and this also confirms that lactose neoglycans can be used as a ligand for E. coli K88 binding.

Inhibition of adhesion of *E. coli* K88+ adhesin to its natural host

One of the main strategies proposed to prevent bacterial infections has been to use soluble glycoproteins
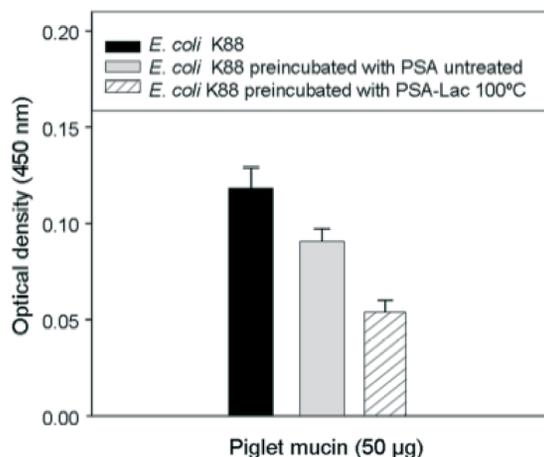

Figure 5. Inhibition of adhesion by *E. coli* K88+ to piglet mucin by PSA-Lac
Mucins (50 µg) were adsorbed in ELISA plate wells, then bacteria (pre-incubated for 1 h with buffer alone, with untreated PSA or with 300 µg of PSA-Lac 100°C) were added to plates. Inhibition was detected as described in Materials and Methods.

that function as ligands for bacterial adhesins to block the interaction of pathogens with host cells (Sharon & Lis, 1989). In this context, an ELLA assay was designed to evaluate this strategy. *E. coli* K88+ was pre-incubated with PSA-Lac 100°C; then allowed to interact with immobilized mucin on an ELISA plate. Under these conditions, recognition of mucin by bacteria was drastically decreased by pretreatment with PSA-Lac. PSA-Lac was able to reduce (approx. 80%) the bacteria adhesion to piglet mucins (Fig. 5). Similar results were obtained with K88ab, ac and ad adhesins (not shown). Untreated PSA presented some non-specific interaction with *E. coli* K88+.

CONCLUSION

This study indicates that non-enzymatic lactosylation of PSA produces neoglycans that are recognized by the galactose-specific *E. coli* K88 adhesins. Further, these glycoconjugates inhibited bacterial adhesion to piglet mucin *in vitro*. *In vivo* studies will be required to support the hypothesis that neoglycans obtained can prevent diarrhea in piglets caused by *E. coli* K88.

Acknowledgements

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