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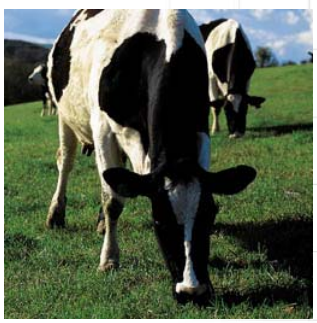
Role of Quiescin Sulphydryl Oxidase in Intestinal Barrier Function

Report to Waikato Medical Research Foundation

FBP 12576

May 2012



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Role of Quiescin Sulphydryl Oxidase in Intestinal Barrier Function

Report to Waikato Medical Research Foundation

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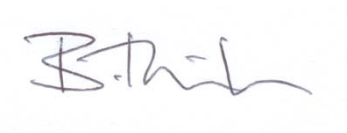

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1. Aim

The aim of this proposal was to explore the hypothesis that milk-derived QSOX enzyme plays a role in intestinal barrier function through generating disulphide bonds that cross-link mucin in the mucus layer. This function may be important to the immature intestine of the neonate, which has only low levels of QSOX compared to the adult (Isaacs *et al.* 1984). If QSOX does play a role in this function then its inclusion in infant formula could provide a benefit to the bottle-fed infant. In addition, QSOX could be used for treating patients with inflammatory bowel diseases that exhibit a bacterially damaged mucous layer.

2. Methods

We explored the effect of QSOX on mucin integrity through examining the chemical, physical and functional properties of mucin and QSOX-treated mucin. In all experiments, mucin was from pig gastric mucus layer and either type II (crude) or type III (purified). In order to break existing disulphide bonds, mucin was treated with beta mercaptoethanol and urea according to Fogg *et al.* (1996), and then exhaustively diafiltered over a 50 Kda ultrafilter against degassed water to remove any reducing agent. Reduced mucin was then freeze-dried and stored between 4 and 8 °C. QSOX was obtained as an enriched fraction from pasteurised bovine milk using cation exchange and heparin affinity chromatography, and ultrafiltration. The final product had an estimated purity of between 12 and 15% by SDS-PAGE and was stored as a freeze-dried powder between 4 and 8 °C. The sulphydryl oxidase activity of this product was confirmed by measuring its ability to oxidise the free thiol DDT according Jaje *et al.* (2007).

2.1 Chemical properties of QSOX-treated mucin

The ability of QSOX to create disulphide bonds in mucin was determined by measuring free thiol content according to Mantle *et al.*(1990). Reduced and non-reduced mucin was dissolved in buffer at 0.625 mg mucin per mL and allowed to hydrate overnight. This was then treated with 0.1 mg/mL QSOX fraction, or with buffer alone, for 2 h at room temperature. Free thiol content was measured by adding 200 uM aldrithiol and measuring the absorbance at 324 nm.

2.2 Physical properties of QSOX-treated mucin

The ability of QSOX to cross-link mucin molecules was determined by examining the molecular size distribution of mucin aggregates using size exclusion chromatography (SEC). Reduced and non-reduced mucin was treated with QSOX as described above. Samples were subjected to SEC using a Sephacryl S300 HR chromatography column,

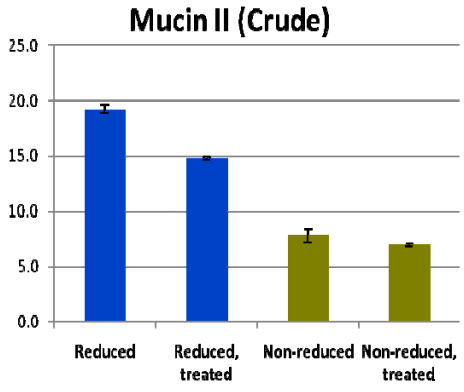
which can separate globular proteins in the molecular weight range 10 kDa to 1500 kDa, and polymeric open structures (e.g. Dextran) in the range 2 to 400 kDa. Chromatography was performed in 10 mM phosphate buffer pH 7.2 containing 150 mM NaCl, and absorbance measured at 280 nm.

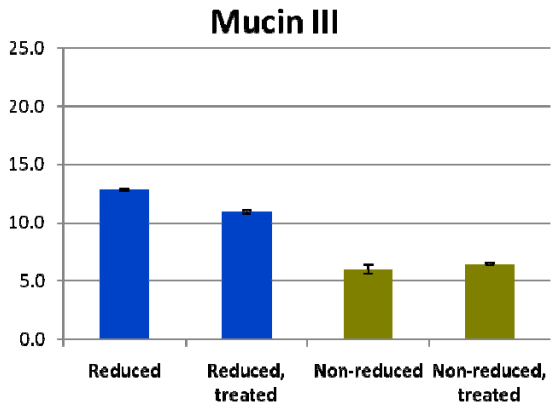
2.3 Permeability of QSOX-treated mucin

The effect of QSOX on the permeability of mucin was determined by measuring the effective diffusion of fluorescently labelled polystyrene microspheres through a hydrated mucin hydrogel. Reduced and non-reduced mucin was treated with QSOX as described above, except that the concentration of mucin was increased to 25 mg/mL to represent levels similar to those observed in the intestine. Fluorescently labelled, biotin tagged microspheres were added to the mucins and mixed in a tube. A proportion was added to the well of a 96-well streptavidin coated fluorescent microplate and left overnight. The degree of diffusion of microspheres through the mucin hydrogel was determined by measuring the level of fluorescent biotin beads captured on the surface of the streptavidin microplate after the mucin had been removed and the well washed with buffer. As control, the effect of QSOX on the capture of fluorescent beads alone was measured.

3. Results

3.1 Disulphide bond formation



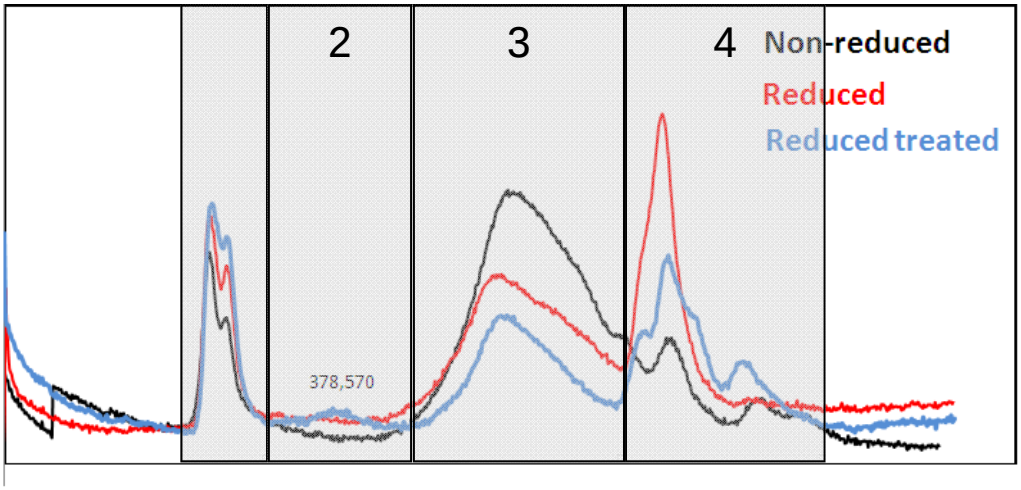


The graphs above show the level of free thiol content of mucin. Reducing mucin with beta mercaptoethanol/urea had the effect of increasing the level of free thiol from around 7.5 nM to 19 nM per mg of mucin II. A similar trend was observed for the more purified mucin III, except the overall levels of free thiol were lower. Treating reduced mucin with QSOX had the effect of lowering free thiol content, or in other words, increasing disulphide bond formation. Although QSOX was able to lower the free thiol

content it was not able to completely restore it to the level of the original non-reduced mucin. Treating non-reduced mucin with QSOX had neglibable effect on free-thiol content.

3.2 Molecular size distribution

The graphs below show the molecular size distribution of mucin. For convenience these chromatograms have been split into four major regions which represent the largest molecular size (region 1) down to the lowest molecular size (region 4). The doublet peak in region 1 probably represents aggregated or networked mucin (first peak) and monomeric mucin (second peak), which has a reported MW of between 1.5 and 2.0 Mda. However, the majority of the protein is represented by lower molecular weight species seen in regions 3 and 4. Reducing mucin with beta mercaptoethanol and urea gave a noticeable drop in region 3 peak size and an increase in lower molecular size species in region 4. There was also a less pronounced but definite increase in the doublet peak in the high molecular size region 1. Treating reduced mucin with QSOX fraction gave a drop in the region 4 peak to a level that was between the non-reduced and reduced profiles. However, instead of observing a rise in the region 3 peak as might be expected, there was instead a drop in this peak, and the appearance of a new peak in region 2 and an increase in the region 1 doublet.



3.3 Permeability of mucin

Attempts were made to measure the permeability of QSOX-treated mucin using fluorescent beads. However, in a control experiment we found that the QSOX fraction gave an unexpected inhibition of the binding of the biotin tagged beads to the streptavidin coated micro-plates. This made it impossible to distinguish between the effect of QSOX on mucin and the effect of QSOX on bead binding alone.

4. Discussion of results

Treating reduced mucin with a milk fraction enriched with active QSOX enzyme can increase the content of disulphide bonds in the mucin. This was observed for both mucin type II preparation, which is a crude extract from pig gastric mucus layer, and for mucin type III preparation, which is a partially purified fraction. QSOX-treated reduced mucin did not obtain the same degree of disulphide bonds as the original non-reduced mucin. There may be several factors why this was not observed. For example, optimal conditions may not have been achieved in our experiments. Perhaps a higher concentration of QSOX, a longer incubation time or a different pH is required for complete oxidation of the free thiols. Alternatively other enzymes or factors may be required.

Treating reduced mucin with the QSOX-enriched milk fraction also had an effect on the molecular size distribution of mucin. Size exclusion chromatography was able to show that low molecular size species decreased and higher molecular size species increased. Mucin has a very complex structure consisting of monomers (with a molecular weight between 1 and 2 million), polymers, aggregates of various sizes, and networks. Also in the mucin are mucin fragments which are believed to be formed from proteolytic cleavage, as well as other non-mucin proteins. These fragments and proteins are probably the lower molecular size species seen in regions 3 and 4 of the chromatograms, which make up the majority of the mucin extract. Some of these fragments have high cysteine content and are believed to be important molecules that for cross-links or bridges between mucin aggregates. What was interesting in our results was the formation of a new molecular size species seen in region 2 for the QSOX treated reduced mucin, but not in any of the other samples tested. This particular peak was also not attributable to any of the proteins present in the QSOX fraction (results not shown). It is not clear what this peak represents but is reasonable to assume that it is composed of cross-linked or aggregated low molecular size species.

We were unable to show the effect of treating mucin with QSOX on the permeability of mucin. This was due to the fact that QSOX, or some other protein in this fraction, had an unexpected inhibitory effect on the binding of biotin tagged beads to the streptavidin coated micro-plate wells. We were therefore unable to adequately use this technique to measure diffusion of the beads through a mucin hydrogel.

5. Conclusion

The results presented here show that the milk-derived enzyme QSOX can form disulphide bonds between mucin molecules and alter the molecular size distribution of mucin aggregates. This is the first piece of evidence that QSOX may play a role in forming or maintaining the structural integrity of mucin is the gut. Since QSOX has been reported to be stable in acid environments, resistant to proteolytic enzymes and to degradation by heat, then it has a good chance of maintaining its activity in the digestive tract of the newborn. Further work will need to establish the effect of QSOX on the permeability of mucin, as well as other properties such as viscosity and resistance to bacterial invasion.

6. References

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7. Financial statement

The $15,000 fund received from WMRF was used for direct labour costs and for lab supplies ($1144) only. Supervision and overhead costs of the project was supported by the Milk Bioactives program funded by the Ministry of Science and Innovation.

8. Summary of the work

This WMRF grant has been used to explore the role of QSOX on mucin functionality. Overall we have had some interesting results which we intend to explore further. We have shown for the first time that mucin, or some component of mucin, is a substrate for QSOX, which is of interest to the scientific community. Our next immediate goal is to explore alternative methods to determine the effect of QSOX on mucin permeability in order to complete our three original research aims. If we find positive results then the three pieces of evidence: chemical, physical and functional, when taken together provide some weight to the hypothesis that QSOX plays a role in mucin functionality. Since QSOX is an extra-cellular secretion in milk, then its purpose in milk is likely to be targeted to the neonate. This therefore raises the question of the importance of providing active QSOX to infants that are bottle-fed on infant formula. It is not known at this stage what the levels of active QSOX are in infant formulae, or what precise effect milk processing has on QSOX in milk.