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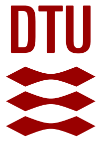

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Optimizing Stability Towards Gut Conditions

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SWINE PLASMA IMMUNOGLOBULINS FOR PREVENTION AND TREATMENT OF POST-WEANING DIARRHOEA: OPTIMIZING STABILITY TOWARDS GUT CONDITIONS

Chris Juul Hedegaard[1], Anne-Sofie Ballegaard[1], Nanna Røjel[1], Marie Bendix Hansen[2], Bodil Kjær Lindved[3], Kirsten Bisgaard Frantzen[4], Lars E. Larsen[1], Allan Lihme[2], and Peter M.H. Heegaard[1]*

1. National Veterinary Institute, Technical University of Denmark, Frederiksberg, Denmark. 2. Upfront Chromatography A/S, Copenhagen, Denmark. 3. KiBif ApS. 4. Multimerics ApS. *contact: PMHH@vet.dtu.dk

Background

Post-weaning diarrhoea (PWD) is a common condition in intensive swine production, resulting in reduced welfare of weaners and economic losses for the farmer as a result of illness, death, treatment costs, e.g. high consumption of antibiotics and zinc oxide.

Aim

1. **Developing feed additives for oral provision for protection against PWD based on natural antibodies (immunoglobulins) derived directly from inexpensive raw materials.**
2. **To increase stability (reducing gut proteolysis) by cross-linking the immunoglobulins (Igs).**

Conclusions

- **The optimal conditions for the Igs were observed to be a moderate multimerisation at pH 9, which confers better pepsin-resistance and increased reactivity towards E. coli O149.**
- **These results suggest that cross-linked Igs could be used for prevention/treatment of PWD and reduce antibiotic consumption.**

Materials & Methods

Immunoglobulin isolation:

Porcine Igs were purified from blood plasma at UpFront Chromatography A/S (Copenhagen) by high-volume Expanded Bed Adsorption with a proprietary adsorbent. Plasma was obtained from a Danish slaughter house. The immunoglobulins were multimerised by controlled periodate oxidation of immunoglobulin-bound carbohydrate (Fig. 1). The multimerisation process was stopped by increasing pH to 12. Cross-coupled Ig-species were analysed by gel filtration using a S300 Sephacryl column and non-reduced 12% Bis-Tris SDS PAGE.

Figure 1: Sodium Periodate ($NaIO_4$) multimerisation

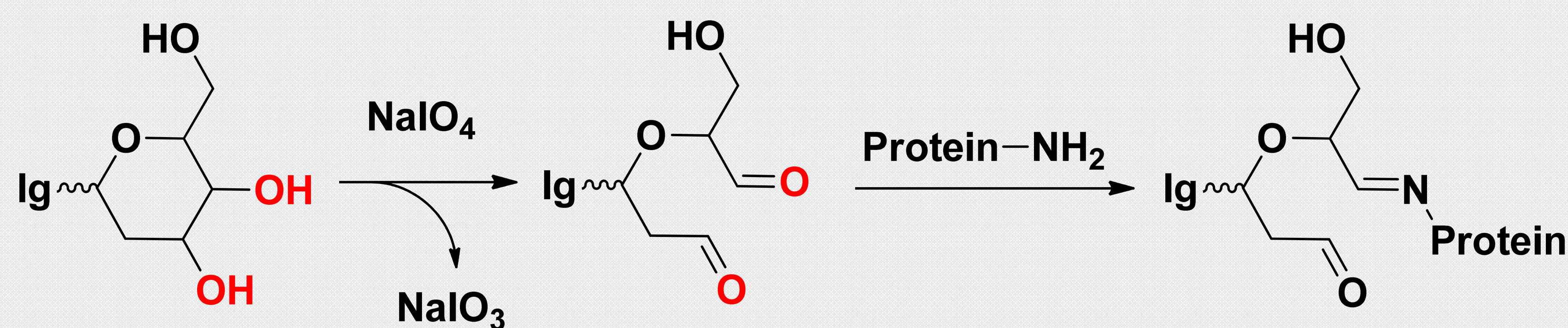

Figure 1. Carbohydrates on immunoglobulins, comprise diols (in red), which are cleavable by the periodate generating aldehydes that in turn can bind to free amines on the polypeptide chain of other immunoglobulins.

ELISA:

A 'Sandwich ELISA' was applied to observe the reactivity of the purified Igs used as the 'bottom' antibody. Extract of Serotype O149 E.coli was used as antigen and a biotinylated rabbit anti-E.coli (AbD Serotec) was used as 'top' antibody.

In vitro gut conditions:

Porcine Igs and 1000 units/ml pepsin were incubated in 50mM sodium acetate pH 3 for 3 hrs. at 37°C whereafter the pepsin was inactivated by adding Na_2CO_3 buffer that increased pH to 9.6.

Next one pepsin treated sample was chosen (pH 9, 0 mM $NaIO_4$), which then was treated either with trypsin, chymotrypsin or with both.

Both proteases were titrated ranging from 887-0.05 u/ml trypsin and 127-0.007 u/ml chymotrypsin. Incubation for 2 hrs. at 37°C. The proteolytical reaction was stopped by addition of the Pefabloc® inhibitor (Sigma-Aldrich).

Results

IMMUNOGLOBULIN MULTIMERISATION:

The degree of Igs-multimerisation was tested at 10, 20 and 40 mM $NaIO_4$ and at different pH values. At low to neutral pH, a tendency towards spontaneous aggregation was observed as these Ig species were eluting early from the gel filtration column (Fig. 2A, pH 6-7) and appear as a high molecular smear on SDS PAGE (Fig. 2B, pH 6-7); in contrast, Igs cross-linked at pH 9 eluted in response to the degree of $NaIO_4$ concentration resulting in a transition from right to left on the chromatographs (Fig. 2A, pH 9) due to the increase in size of the multimers (Fig. 2B, pH 9).

Figure 2.

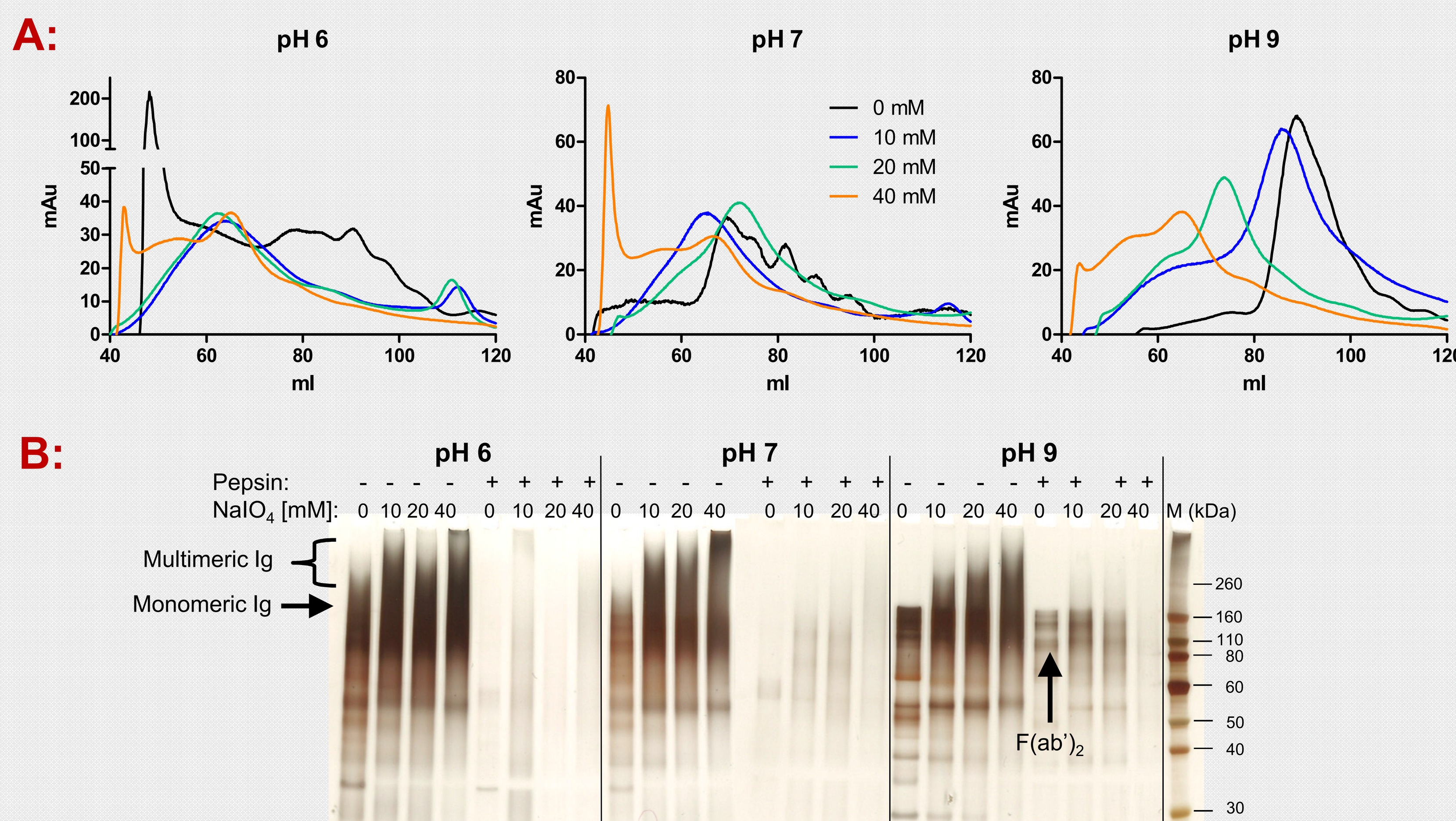

Figure 2. (A): Immunoglobulin-multimerisation carried out at different pH and periodate concentrations (coloured lines) as compared to non-periodate aggregates (black line) on Sephacryl S300 gelfiltration. **(B):** The samples (grouped by pH of cross-coupling reaction) visualised by Silver stained non-reduced SDS PAGE. From left the non-cross-coupled Igs; then Igs multimerised at 10mM to 40 mM $NaIO_4$ either without or with 1000 unit pepsin.

GUT CONDITIONS:

In general, pepsin degraded the porcine Igs extensively (Fig. 2B, pepsin) and especially Igs that had been subjected to periodate oxidation at low/neutral pH conditions were less stabile in the presence of pepsin than those samples that had been periodate treated at pH 9. Furthermore, greater multimerisation also resulted in lower stability (Fig. 2B, pepsin).

Periodate-induced porcine Ig multimerisation carried out at low to neutral pH or at high periodate concentrations (20 and 40 mM) did not favour E. coli reactivity (Figure 3A) in comparison to periodate oxidation done at 10 mM and pH 9, which on the contrary increased Ig-reactivity towards E. coli (Figure 3A, 10 mM).

Digestion of the pepsin-treated 'pH 9 sample' with trypsin and chymotrypsin revealed that trypsin, in contrast to chymotrypsin, does not digest the $F(ab')_2$ well (Fig. 3B).

Figure 3.

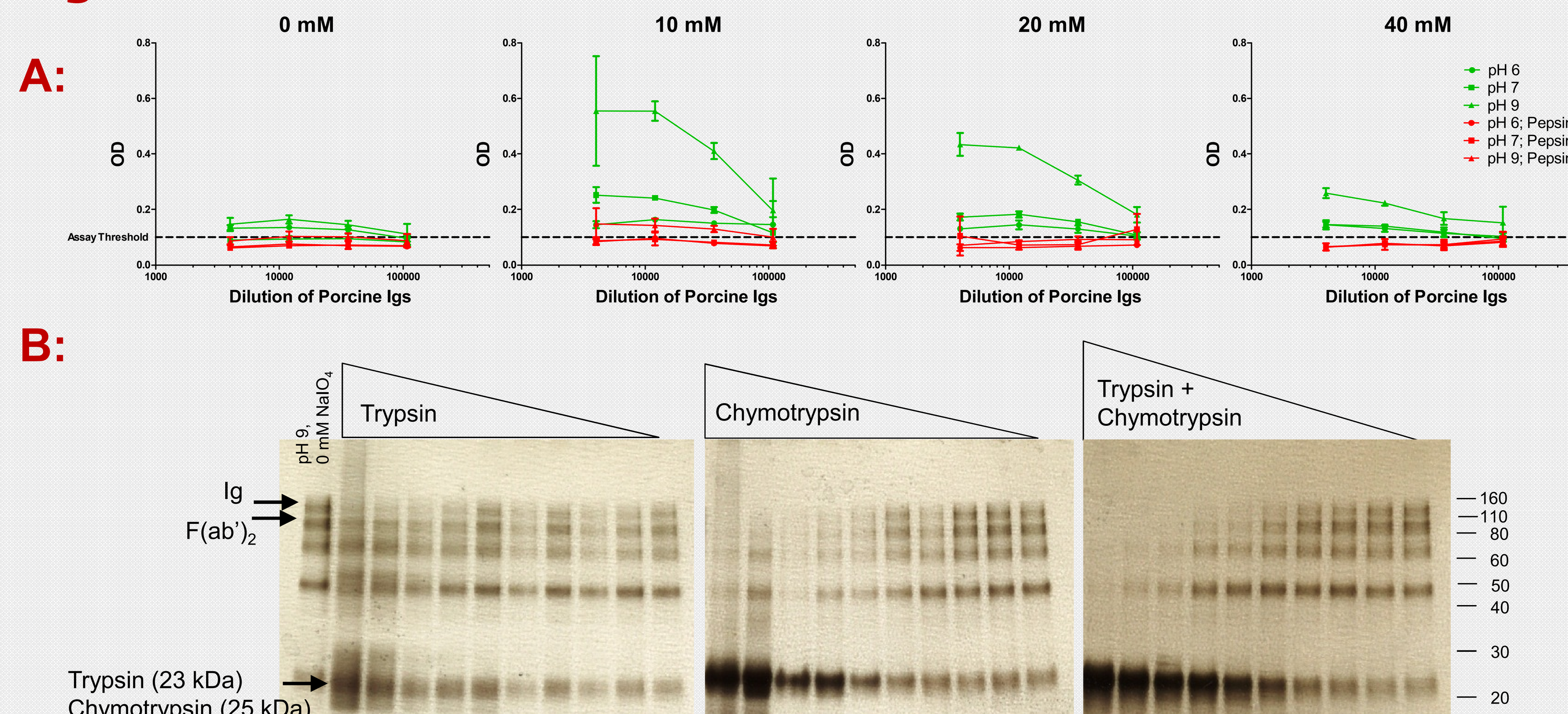

Figure 3. Protease digestions of the porcine Igs. (A): After cross-coupling (green) and pepsin digestion (red) these Igs were used as the bottom antibodies in a sandwich ELISA. Lysate from serotype O149 E. coli was used as antigens and a biotinylated rabbit anti-E. coli antibody was used as top antibody/for development. **(B):** Three-fold titration of trypsin or chymotrypsin alone or both proteases together on the pepsin treated non-multimerised pH 9 sample (see fig. 2B, arrow). Trypsin ranged from 887 to 0.05 u/ml and chymotrypsin from 127 to 0.007 u/ml.