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# The physicochemical characterisation of pepsin degraded pig gastric mucin

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29 **Abstract**

30 Mucins are the main macromolecular components of the mucus secretions that cover the oral cavity,  
31 gastrointestinal and urogenital tracts of animals. The properties of the mucus secretions are  
32 therefore directly correlated with the physicochemical properties of mucin glycoproteins. In this  
33 study, mucins were obtained from pig gastric mucous after digestion with pepsin at 37 °C for 4  
34 hours, these mucins were characterised in terms of compositional and hydrodynamic properties.

35  
36 Compositional analysis showed that this mucin contains protein (15%), carbohydrates (55%) of  
37 which the constituents are: fucose (4%), galactose (9%), glucosamine (55%), glucosamine (33%)  
38 and sialic acid (2%). The latter component gives the mucin polymer a pH-dependant negative  
39 charge, with a  $\zeta$ -potential of -3 mV at pH 1.2 up to -11 mV at pH 7.4. The weight average molar  
40 mass was  $\sim 1 \times 10^6$  g/mol and intrinsic viscosity was  $\sim 0.42$  dL/g although there was a small pH  
41 dependency due to the polyelectrolyte behaviour of the polymer. The measurements of viscosity  
42 versus shear rate showed shear thinning behaviour and the critical overlap concentration was  
43 determined to be 10-11% w/v indicating a compact structure. Knowledge of these properties is  
44 fundamental to the understanding interactions of mucins, with for example, novel drug delivery  
45 systems.

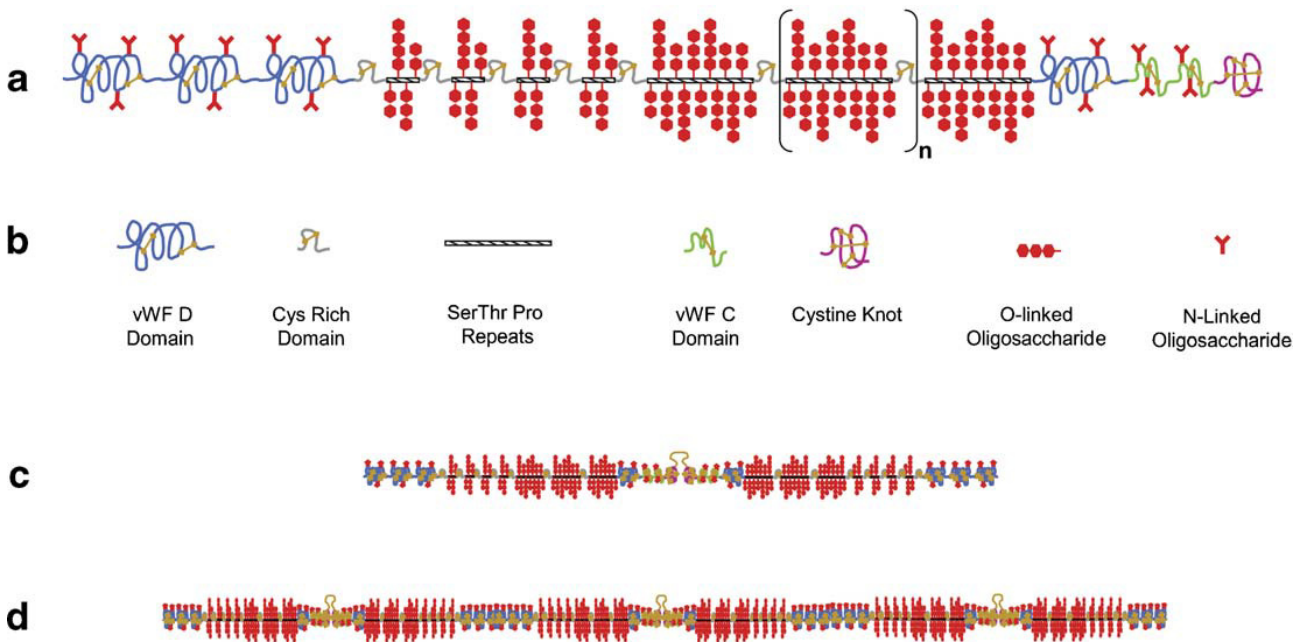
46  
47 **Keywords:** pepsin degraded mucin; physicochemical properties; compact conformation

48 **Highlights:**

- 49       • The physicochemical properties of extensively degraded mucin were investigated
- 50       • Mucin consisted of fucose, galactose, glucosamine, glucosamine and sialic acid
- 51       • Weight average molar mass was  $1 \times 10^6$  g/mol and intrinsic viscosity was  $\sim 0.42$  dL/g
- 52       • Critical overlap concentration was determined to be 10-11 % w/v
- 53       • Data is consistent with a weak polyelectrolyte behaviour and compact conformation

54 **1. Introduction**

55 Mucins are the main macromolecular components of the mucus secretions that cover the oral cavity  
56 and the respiratory, gastrointestinal and urogenital tracts of animals. Moreover, they provide  
57 protection for the delicate exposed epithelial surfaces and are responsible for the viscoelastic  
58 properties of the mucosal secretions [1]. The polymeric structure of the component mucins are  
59 directly correlated with the protective properties of the mucus gel [2]. Mucins are large,  
60 extracellular, abundant, filamentous molecules [3] with the molecular weight range from  $5 \times 10^5$  up  
61 to  $2 \times 10^7$  g/mol [4]. Mucin structures are stabilized by inter-chain disulphide bonds [5, 6]. The  
62 mucin protein core contains highly glycosylated regions comprising of 80 % carbohydrates  
63 primarily of *N*-acetylgalactosamine (GalNac), *N*-acetylglucosamine (GlcNac), galactose (Gal),  
64 fucose (Fuc) and sialic acid (*N*-acetylneuraminic acid, Neu5Ac) and traces of sulphate ( $\text{SO}_4^{2-}$ ) and  
65 mannose (Man) (**Figure 1**) [7] which are therefore highly resistant to proteolysis and whereas the  
66 regions which are sparsely glycosylated or non-glycosylated regions are subsequently much more  
67 susceptible to proteolysis [5, 8, 9]. Mucin is negatively charged due to the presence of sulphate  
68 esters and sialic acid. The oligosaccharide chains consisting of 5–15 units show moderate branching  
69 and are attached to the protein core by O-glycosidic linkages to the hydroxyl side chains of serine  
70 and threonines and arranged in a “bottle brush” shape about the protein core [7, 10]. Colonic  
71 mucin in either its polymeric, reduced (with mercaptoethanol) or digested (with papain) forms have  
72 been reported to adopt random coil conformations [11-13] as was proposed by the general model  
73 [14].



74

75 **Figure 1. (a)** A schematic drawing of the pig gastric mucin monomer consisting of glycosylated  
76 regions flanked by regions with relatively little glycosylation. **(b)** The symbols indicate the different  
77 domains in the sketch in **(a)**. (This representation is based in part on **Figures 1 and 2** [3]. The  
78 cysteine rich regions contain domains that are similar to von Willebrand factor (vWF) C and D  
79 domains, and C-terminal cysteine knot domains which have been shown to be involved  
80 dimerization and subsequent polymerisation to form larger multimers. The bottom of the figure  
81 shows **(c)** a dimer formed by two monomeric subunits linked via disulfide bonds in the non-  
82 glycosylated regions and in **(d)** dimers that are further disulfide linked to form higher multimers.  
83 This gives rise to the high molecular weight and polydispersity of secretory mucins. Polymers of  
84 greater than 16-mers have been described in MUC5AC from human airway secretions by [15]. (The  
85 bottom part of the figure is adapted from **Figure 8** in [15]. Figure reprinted with permission from  
86 [7].

87

88 As the rheological interactions of mucoadhesive polymers will be affected not only by the chemical  
89 structure of mucins but also by the way in which the mucin has been prepared [16]. The aim of this  
90 article is to fully characterise extensively degraded pig gastric mucin with the respect to  
91 compositional and hydrodynamic properties to underpin the understanding of mucin interactions  
92 with polysaccharide based drug delivery systems. Furthermore, any information about this material  
93 could open up opportunities for novel application areas of digested mucins.

94

## 95 **Materials and methods**

96 Glucose, sodium tetraborate (borax), sodium acetate, phenyl phenol, glacial acetic acid, sodium  
97 acetate trihydrate, trifluoroacetic acid, sialic acid, periodic acid, sodium arsenite, bovine serum  
98 albumin (BSA), Bradford reagent, n-butanol, hydrochloric acid, sodium hydroxide, sulphuric acid,  
99 thiobarbituric acid and sodium chloride were all obtained from Sigma-Aldrich (Gillingham, UK).  
100 Extensively degraded pig gastric mucin was obtained from Biofac A/S (Kastrup, Denmark). All  
101 materials were used without any further purification.

102

### 103 **2.2. Preparation and purification of digested gastric mucins**

104 The mucins were prepared as a by-product from large scale preparation of pharmaceutical quality  
105 pepsin at Orthana Kemiske Fabrik A/S (part of the Biofac group) in Copenhagen, Denmark. Red  
106 linings from porcine stomachs were obtained from abattoirs in the US (Farmland). These were kept  
107 frozen (-18 °C) until they were taken into use in the production area. First, approx. 1000 kg of

108 frozen linings were minced in a large meat mincer (screen 18 mm). The minced raw material was  
109 transferred into a stirred tank before adding 100 kg of RO water. Then, the pH was adjusted to 2.0  
110 using concentrated HCl before heating to 38 °C. After 4.5 h, the pH was adjusted to 2.8 using  
111 concentrated NaOH. The process liquid was transferred to a precipitation tank and cooled down to -  
112 5 °C. The crude mucin was then precipitated with 97 % acetone added slowly until 61 % w/w. The  
113 precipitation liquid was held at -5 °C and mixed using mild agitation for 30 minutes. The process  
114 liquid was then separated on a Flotweg decanter (1500 rpm inner speed, 6000 rpm outer speed) into  
115 liquid and solid phases where the latter contained fat and mucins. The precipitate was solubilized by  
116 adding approx. 5 volumes of water. Remnants of acetone were evaporated off at 40 °C under  
117 vacuum. Subsequently, the liquid was left to sediment for 3 days before pumping the top phase  
118 (clear liquid) out. The crude mucin was then filtered on a Seitz Orion plate and frame filter press  
119 three times using cellulose and filter aid based filter plates (first T2600, T1000 and finally K250, all  
120 from Seitz, Pall Corporation, New York, USA) coated with filter aid (Hyflo Super Cel). The mucin  
121 was then concentrated to 5 % solid content and washed with 3 volumes of RO water before pH  
122 adjustment to 3-4 and subsequently frozen at -18 °C and lyophilized.

123

## 124 **2.3. Chemical characterisation of gastric mucin**

### 125 **2.3.1. Determination of total carbohydrate using a phenol sulphuric acid assay [17]**

126 Total carbohydrates in the mucin sample were colorimetrically determined by *m*-hydroxydiphenyl  
127 method. Firstly, a stock solution of glucose (200 mg/L) was prepared and from this stock solution,  
128 standard solutions with concentrations of 0 - 100 mg/L were prepared, then the glucose test was  
129 performed by taking 400 µL from the standard solutions. Two ml of 0.5 % borax in concentrated  
130 sulphuric acid was added and then incubated at 100 °C in water bath for 5 min to which 40 µL of  
131 0.15 % 3-phenylphenol (in 1 M sodium hydroxide) was added and incubated for 5 min. The  
132 absorbance for each standard and the sample was measured at 520 nm using Shimadzu UV-160A  
133 UV-vis spectrophotometer. The blank for the sample was prepared by taking 400µL of the sample,  
134 2 mL of deionised water and 40 µL of 0.15 % 3-phenylphenol while the blank for the standard was  
135 prepared by taking 400 µL of deionised water, 2 mL of 0.5 % borax in concentrated sulphuric acid  
136 and 40 µL of 0.15 % 3-phenylphenol.

137

138 **2.3.2. Determination of total protein using Bradford assay**

139 Five dilutions of Bovine Serum Albumin (BSA) standard with a range of 5 to 100 mg/L were  
140 prepared. 30  $\mu$ L of each mucin solution (250 mg/L) and the standard solutions were added to  
141 separate test tubes. The blank was prepared using 30  $\mu$ L ultrapure water instead of standard solution  
142 or mucin sample. Bradford reagent (1.5 mL) was added to each tube and mixed well. The samples  
143 were incubated at room temperature for 10 min. The absorbance measurements of the mucin  
144 samples were recorded at 595 nm and the concentration of protein was calculated from a standard  
145 curve and expressed as a percentage by weight of mucin.

146

147 **2.3.3. Determination of the constituent sugars by high-performance anion-exchange**  
148 **chromatography with pulsed amperometric detection (HPAEC-PAD)**

149 Mucin (2.0 mg in duplicate) was dissolved in 2 mL of deionized water in separate pressure tubes.  
150 Concentrated trifluoroacetic acid (0.85 mL) was then added to each sample solution using a  
151 micropipette. The pressure tubes were then placed in a heating block for 2 hours at 120 °C. After 2  
152 hours the samples were evaporated to dryness under a stream of nitrogen gas at 65 °C for 1 hour.  
153 The dried samples were reconstituted with 2 mL of deionized water and the sample diluted 10 times  
154 prior to HPAEC-PAD analysis. Neutral sugars, amino sugars and sialic acid composition were  
155 analysed using a Dionex ICS-5000 HPAEC-PAD system (Thermo Fisher, Loughborough, UK). A  
156 0.5 mL/min flow rate was used the first 12 minutes at a concentration of 10 mM NaOH this was  
157 then followed by a 0.05 minute step to change from 0-17 % 1 M sodium acetate in 150 mM NaOH  
158 and the remainder of the run was continued at 17 % 1 M sodium acetate in 150 mM NaOH to elute  
159 any uronic acids present. A pre-run equilibration step of 10 minutes using 200 mM NaOH followed  
160 by 20 minutes of 10 mM NaOH was used to regenerate the column prior to each injection.

161

162 **2.3.4. Determination of sialic acid using sialic acid assay**

163 Sialic acid determination was achieved by using the method of [18]. 10 mg of mucin was  
164 hydrolysed in 2 mL 100 mM H<sub>2</sub>SO<sub>4</sub> at 80 °C for 1 h to release sialic acids (in duplicate), then  
165 neutralised with 1M NaOH (45  $\mu$ L). The samples were incubated with 250  $\mu$ L periodic acid  
166 solution (25 mM in 62.5 mM H<sub>2</sub>SO<sub>4</sub>) at 37 °C for 30 min. The reaction was concluded by adding  
167 0.2 mL sodium arsenite (2 % in 0.5 M HCl), left for 3 min before adding 2 mL thiobarbituric acid  
168 (0.1 M, pH 9.0). The solutions were heated in a boiling water bath for 7.5 min then cooled in ice  
169 water and mixed with 5 mL of n-butanol /concentrated HCl solution (95:5, v/v), shaken and the  
170 absorbance of the butanol layer was measured at 550 nm. The concentration of sialic acids was

171 calculated from a standard curve constructed with N-acetyl neuraminic acid (1–500 µg/mL) and  
172 expressed as a percentage by weight of mucin.

173

## 174 **2.4. Physical characterisation of gastric mucin**

### 175 **2.4.1. Determination of weight-average molecular weight by size-exclusion** 176 **chromatography coupled to multi-angle laser light scattering (SEC-MALS)**

177 A 0.5 % w/v of mucin was analysed by size exclusion chromatography which was carried out at  
178 ambient room temperature on a PL aquagel guard column (Polymer Labs, Amherst, U.S.A.) which  
179 was linked in series with PL aquagel-OH 60, PL aquagel-OH 50 and PL aquagel-OH 40 (Polymer  
180 Labs, Amherst, U.S.A.) and was eluted with distilled water at a flow rate of 0.7 mL/min. The eluent  
181 was then detected online firstly by a DAWN EOS light scattering detector (Wyatt Technology,  
182 Santa Barbara, U.S.A.) and a REX differential refractometer (Wyatt Technology, Santa Barbara,  
183 U.S.A.). The refractive index increment,  $dn/dc$  was taken to be 0.150 mL/g.

184

### 185 **2.4.2. Determination of intrinsic viscosity**

186 Appropriate concentrations of mucin were prepared (0.025 – 0.2 % w/v) at pH 1.2, 4.4 and 7.4,  
187 respectively. The measurements were performed with a Cannon capillary viscometer size 50 at 25  
188 °C. The relative ( $\eta_{rel}$ ) and specific viscosities ( $\eta_{sp}$ ) were calculated as described in equations 1 and  
189 2, respectively:

190

$$191 \quad \eta_{rel} = \left( \frac{t}{t_0} \right) \quad (1)$$

192

$$193 \quad \eta_{sp} = \eta_{rel} - 1 \quad (2)$$

194

195 where  $t$  is the average flow time of the solutions at each concentration,  $t_0$  is the flow time for the  
196 appropriate solvent [19]. Measurements were made at different concentrations and extrapolated to  
197 infinite dilution using both the Huggins and Kraemer approaches [20, 21]:

198

$$199 \quad \frac{\eta_{sp}}{c} = [\eta](1 + K_H [\eta]c) \quad (3)$$

200



201 
$$\frac{\ln(\eta_{rel})}{c} = [\eta](1 - K_K [\eta]c) \quad (4)$$

202

203 where the intrinsic viscosity  $[\eta]$  is taken as the mean of the intercepts from equations (3) and  
204 (4) and  $K_H$  and  $K_K$  are the Huggins [20] and Kraemer [21] constants respectively.

205

### 206 **2.4.3. Determination of the critical coil overlap ( $c^*$ )**

207 A stock solution mucin (40 w/v %) was prepared by dissolving 40 g of mucin in 100 mL of  
208 deionized water. Once fully dissolved, the stock solution was diluted to appropriate range of  
209 concentrations (1 – 40 %). Mucin solutions of the same concentrations were also prepared at pH 1.2  
210 and 7.4 pH by adjusting the pH with 0.1 M HCl and 0.1 M NaOH respectively. The viscosities at  
211  $130 \text{ s}^{-1}$  were measured using cone plate 55 mm geometry on a Bohlin Gemini HR Nano Rheometer  
212 at  $37 \text{ }^\circ\text{C}$ .

213

### 214 **2.4.4. Determination zeta potential, $\zeta$**

215 A solution of mucin (0.5 % w/v at pH 1.2, 4.4 and 7.4) was prepared by dissolving 0.5 g of mucin  
216 in 100 mL of deionized water and the pH was adjusted accordingly with 0.1 M HCl or 0.1 M  
217 NaOH. The zeta potential of the three samples was determined using Malvern Zetasizer NANO-Z  
218 (Malvern Instruments Limited, Malvern, UK). Measurements in triplicate were performed by using  
219 a folded capillary cell at  $25.0 \pm 0.1 \text{ }^\circ\text{C}$  and refractive index was set at 1.450.

220

### 221 **2.4.5. Rheological study**

222 Measurements of viscosity vs. shear rate were performed at  $37 \text{ }^\circ\text{C}$  on 7 % and 15 % w/v mucin  
223 samples prepared at pH 1.2, 4.4 and 7.4 across shear rates ranging from  $1 \text{ s}^{-1}$  to  $1000 \text{ s}^{-1}$  using cone  
224 and plate 55 mm geometry fitted to a Bohlin Gemini Rheometer (Malvern Instruments, UK). Small  
225 deformation oscillatory measurements were also performed on these solutions (7 % and 15 % at pH  
226 1.2, 4.4, and 7.4) to monitor the viscoelastic behaviour of the mucin using the same rheometer as in  
227 the viscosity measurements but using a double gap geometry to minimise signal to noise ratio.  
228 Measurements of storage modulus ( $G'$ ) and loss modulus ( $G''$ ) were taken at frequencies from 0.1  
229 rad/s to 10 rad/s to ascertain mechanical spectra of the gels at an isothermal temperature of  $37 \text{ }^\circ\text{C}$   
230 and at a fixed strain of 2 %. Measurements were performed in triplicate and mean values plotted.

231

232 **3. Results and discussion**

233 **3.1. Chemical characterisation of gastric mucin**

234 A phenol sulphuric acid assay was used to determine the total carbohydrate of the mucin samples  
235 relative to glucose standards. The mucin had a total carbohydrate content of 55 % as glucose  
236 equivalents and a total protein content of 15 % when using bovine serum albumin as a standard  
237 (**Table 1**). It is noted that the recovery for total protein and total carbohydrate does not equate to  
238 100 % this may be due to the use of glucose as standards, as the response to the assay varies with  
239 different monosaccharides [17]. The mucin also contains ~ 10 % moisture.

240

241 **Table 1:** Some physicochemical properties of the gastric mucin

Property	Measurement
<b>Total carbohydrate, % (as glucose equivalents)</b>	55 ± 1
<b>Fucose, mol %</b>	4 ± 1
<b>Galactose, mol %</b>	9 ± 1
<b>N-acetylgalactosamine, mol %</b>	55 ± 1
<b>N-acetylglucosamine, mol %</b>	33 ± 1
<b>Sialic acid, %</b>	1.7 ± 0.1
<b>Total protein, % (relative to BSA standards)</b>	15 ± 1
<b>M<sub>w</sub>, 10<sup>6</sup>g/mol</b>	1.04 ± 0.05
<b>M<sub>w</sub>/M<sub>n</sub></b>	5.5 ± 0.5
<b>r<sub>g,z</sub>, nm</b>	31 ± 6

242

243 Constituent sugar analysis using HPAEC revealed the presence of Fuc, Gal, GalN and GlcN (**Table**  
244 **1**) which are consistent with previous results [22]. We were unable detect any sialic acid using this  
245 method, but it has been determined by an alternative method (sialic acid assay – section **2.3.4**) to be  
246 1.7 %.

247

248 **3.2. Molecular weight**

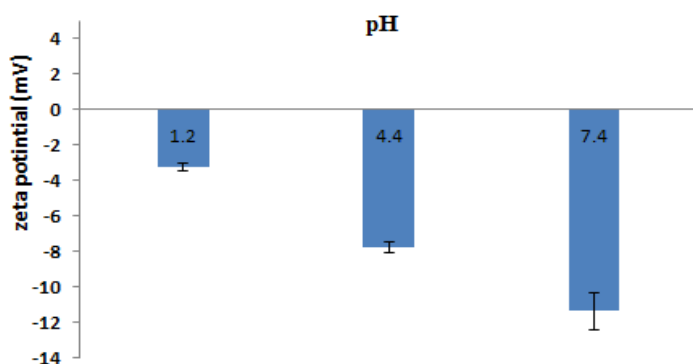
249 The weight-average molecular weight as measured by size-exclusion chromatography coupled to  
250 multi-angle laser light scattering (SEC-MALS) was found to be 1.04 x 10<sup>6</sup> g/mol which is in  
251 general agreement with previous estimates [12] and demonstrates that the enzymatic digestion has  
252 resulted in a large reduction in molecular weight as typically non-degraded pig gastric mucin has a  
253 weight-average molecular weight of 5 – 9 x 10<sup>6</sup> [11, 12, 23]. MALS can also give an approximation

254 of the radius of gyration ( $r_{g,z}$ ), which was estimated to be 31 nm. This is indicative of compact  
255 structure and is of the size of typical T-domains [24].

256

### 257 3.3. Zeta potential

258 Measurements of  $\zeta$ -potential were taken as an indirect measurement of surface charge and were  
259 performed on the samples at pH 1.2, 4.4 and 7.4. **Figure 2** shows a negative charge for all the  
260 samples tested with a progressive negative charge increase with increasing pH. This may be  
261 attributed to the presence of the carboxylic acid group in sialic acid. Studies on *native* pig gastric  
262 mucin have previously shown an isoelectric point at  $\sim$  pH 2-2.5 [25] and sialic acid has a  $pK_a$  of 2.6  
263 [26].



264

265 **Figure 2.** Zeta potential of samples of gastric mucin (0.5 % w/v) prepared in deionised water and  
266 pH adjusted to pH 1.2, 4.4 and 7.4.

267

### 268 3.4. Intrinsic viscosity

269 The weight-average intrinsic viscosity,  $[\eta]_w$  was found to be 0.42 – 0.44 dL/g which is in general  
270 agreement with previous estimates [27] and is also consistent with the reduction in molecular  
271 weight. A weight-average intrinsic viscosity of 0.42 – 0.44 dL/g coupled with a weight-average  
272 molecular weight of  $1.04 \times 10^6$  g/mol suggests a compact conformation [28, 29].

273

### 274 3.5. Critical overlap concentration ( $c^*$ )

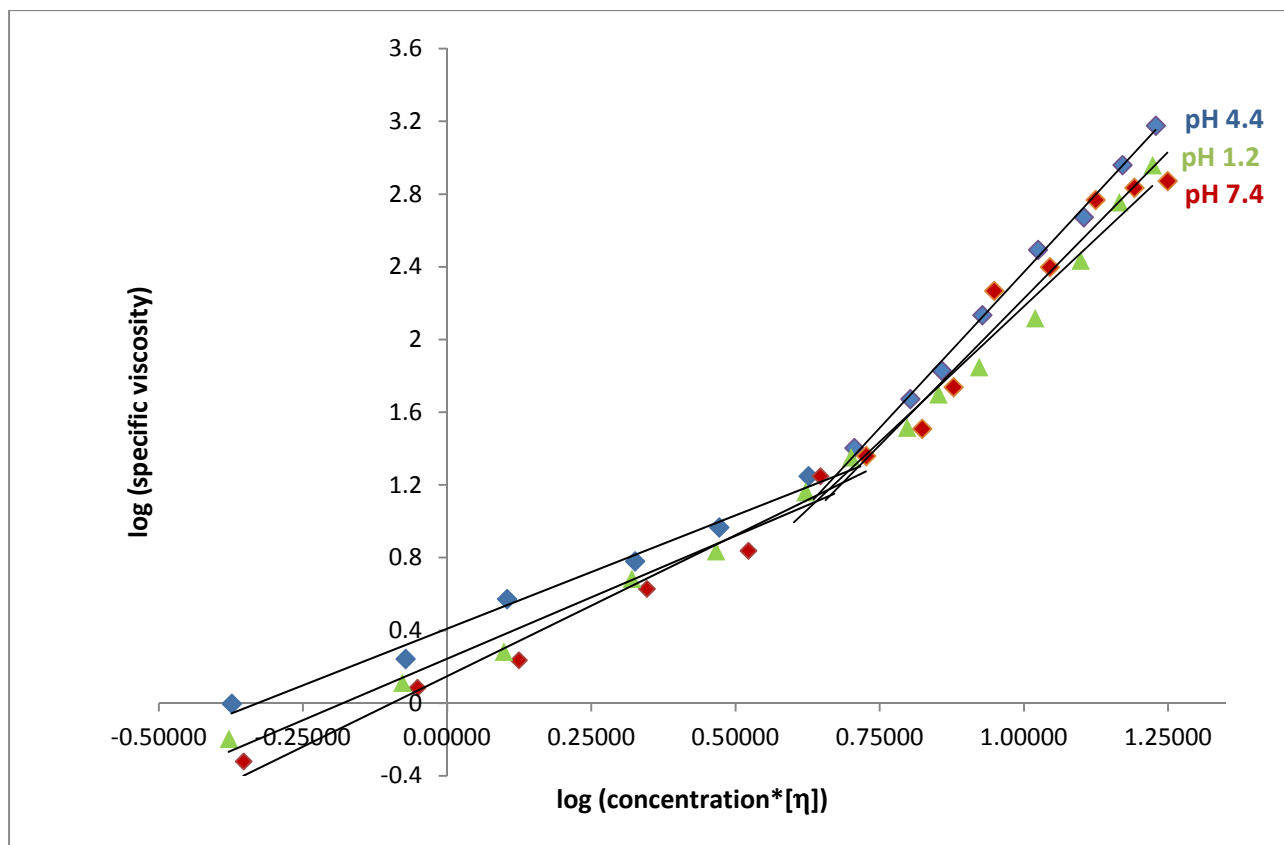
275 In a dilute solution, random coils of polymer are spaced from each other. With increasing the  
276 concentration of polymer solution, the distance between the coils become smaller and coils starts to  
277 overlap and entangle. The concentration at which the individual polymer coils starts to overlap and  
278 entangle is termed overlap concentration ( $c^*$ ) [30]. Above  $c^*$ , viscosity increases rapidly with  
279 increasing concentration [31] as the chains of polymer interpenetrate with each other. This leads to  
280 difficulty in studying the characteristics of individual chains in solution [30]. Entanglement

281 characteristic is affected by the concentration of the solution and the hydrodynamic radius of the  
282 polymer, which for polyelectrolytes is dependent on pH and ionic strength [32]. As the entangling  
283 of polymer coils depend on their molecular size (hydrodynamic volume), chain stiffness and  
284 excluded volume effects [32]. Where the latter is probably very important for branched mucins.  
285 Therefore a decrease in molecular weight would be expected to have high impact on the viscoelastic  
286 properties of degraded mucin solutions [31].

287

288 It has been found that, at a mucin concentration of ~11 % (w/v) the mucin chains start to overlap  
289 (**Figure 3**) which agrees with the generalised theory where  $\log c^*[\eta] \sim 0.6$  and  $\log \eta_{sp} \sim 1$  [33]. The  
290 relatively high  $c^*$  is consistent with the molecular weight of the mucin being relatively low  
291 (compared with native mucins) and in this case adopting a compact conformation (**Table 2**) for  
292 example pullulan (a random coil type polysaccharide) of the same molar mass would be expected to  
293 have an intrinsic viscosity of ~ 2 dL/g [34] under similar conditions and a polyanion like pectin  
294 (semi-flexible coil) would be expected to be ~ 20 dL/g [35]. There is little influence of the pH  
295 change on either intrinsic viscosity or  $c^*$ , probably due to excluded volume effects between the  
296 different branches on each mucin molecule forcing the chains into an expanded conformation  
297 giving less possibility for relaxation of the chain stiffness even when electrostatic repulsion along  
298 the chains decreases with lower pH due to fewer of the carboxylic acid moieties of sialic acid being  
299 deprotonated [32]. Pepsin degraded pig gastric mucin therefore appears to be similar hydrodynamic  
300 size to the T-domains produced using trypsin digestion [14]. The values of  $c^*$  measured here are  
301 higher than what has previously been suggested 0.2-0.4 % [31], 2.5 % for Muc5ac and 3 % for  
302 Muc2 [36] and again, this is probably due to the specific pepsin degradation during processing.

303



304

305 **Figure 3.** Intersection of two curves of log concentration\*[ $\eta$ ] versus log specific viscosity. The  
 306 means slopes of the plots are 1.4 and 3.2 for the dilute and concentrated regimes, respectively.

307

308 **Table 2.** The effect of pH on some physical properties of digested porcine gastric mucin

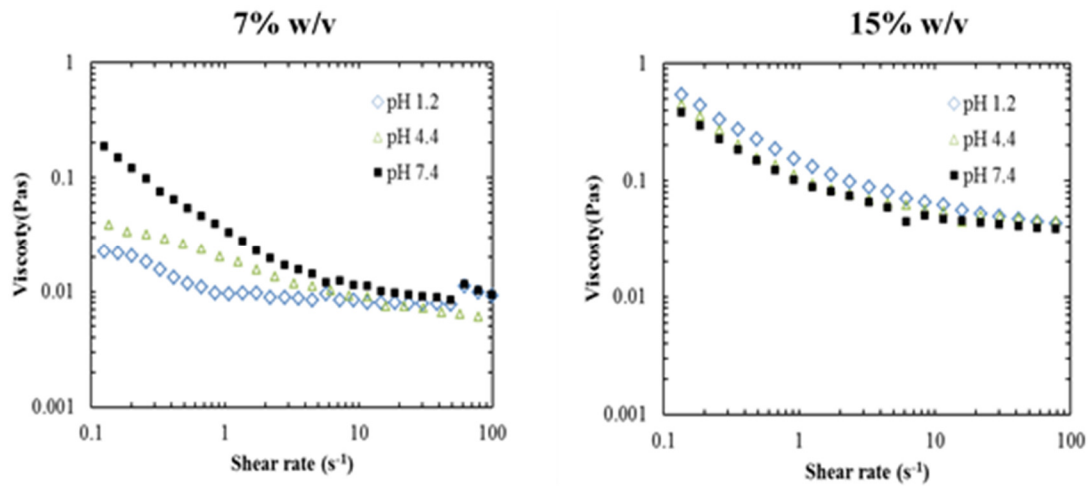
Property	pH		
	pH 1.2	pH 4.4	pH 7.4
[ $\eta$ ], dL/g	$0.416 \pm 0.003$	$0.426 \pm 0.004$	$0.443 \pm 0.012$
$c^*$ , g/dL (%)	$11.0 \pm 0.1$	$11.1 \pm 0.1$	$10.3 \pm 0.3$
$\zeta$ -potential, mV	$-3.4 \pm 0.2$	$-7.8 \pm 0.3$	$-11.4 \pm 1$

309

### 310 3.6. Rheological study

311 All mucin samples showed typical shear-thinning behaviour with viscosity decreasing with  
 312 increasing shear rate (**Figure 4**). The 7 % w/v sample (below  $c^*$ ) at pH 7.4 showed a distinctly  
 313 higher viscosity compared with the samples at acidic pH. This can be explained by the mucin  
 314 molecules becoming more extended at a higher pH causing an increase in entanglement and hence  
 315 viscosity. Zeta potential measurements showed that the charge increased with increasing pH which  
 316 would likely be the cause of a more extended conformation due to an increased intra molecular

317 repulsion which is consistent with increased viscosity. This difference is not apparent at 15 % w/v  
318 (above  $c^*$ ) due to the increase in polymer concentration, the intermolecular entanglements increase  
319 and dominate the viscosity effect of intra molecular repulsion. The relatively low viscosity suggests  
320 that the hydrodynamic size of the mucins is likely to be relatively small due to compact structure  
321 and/ or branching.

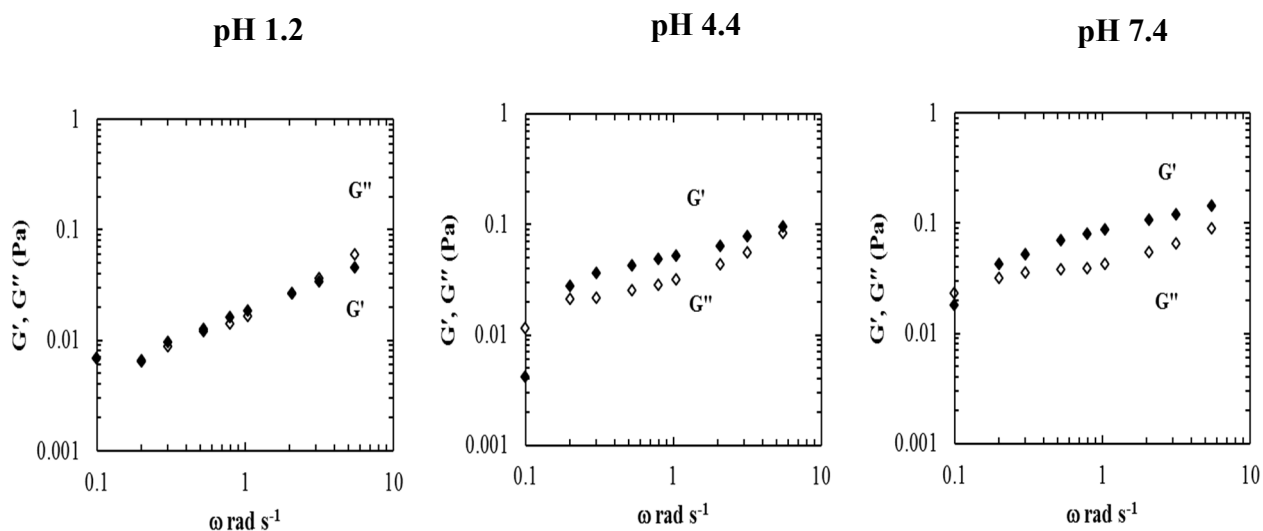


322  
323 **Figure 4.** Viscosity vs. shear rate of 7 % (w/v) and 15 % (w/v) mucin samples at varying pH  
324 measured at 37 °C.

325

### 326 3.7. Dynamic mechanical measurements

327 Small deformation oscillatory measurements of elastic ( $G'$ ) and viscous modulus ( $G''$ ) were  
328 undertaken to monitor the viscoelastic behaviour of the mucin using a Bohlin Gemini rheometer  
329 fitted with a double gap geometry. Amplitude sweeps were performed to ascertain the linear  
330 viscoelastic region of the samples. To reveal the mechanical spectra of the mucin, measurements  
331 were taken over a frequency range of 0.1 to 10 rad/s at 2 % strain at 37 °C. **Figure 5** highlights the  
332 difference in mechanical spectra of 7 % w/v mucin at pH 1.2, 4.4 and 7.4. These results show a  
333 slight increase in moduli at pH 4.4 and 7.4 compared with the values obtained at pH 1.2.  
334 Interestingly this contradicts the results on *native* pig gastric mucin which exhibits a pH dependent  
335 sol-gel transition when pH is reduced to  $\leq$  pH 4 [37], although this would also be expected to be  
336 concentration dependent [36]. Again this is attributed to the polymer extending as the pH increases  
337 allowing a higher degree of polymer entanglement.



338

339

340 **Figure 5.** Mechanical spectra of 7 % (w/v) mucin samples at varying pH measured at 37 °C.

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#### 4. Conclusions

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#### 5. Acknowledgements

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#### 6. Conflict of interest statement

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Drs. Tømmeraas and Ronander are, or were at the time of the study, employees of Biofac A/S.

361 **7. References**

- 362 [1] M.U. Adikwu, *Tropical Journal of Pharmaceutical Research*, 5 (2006) 581-582.
- 363 [2] L.A. Sellers, A. Allen, E.R. Morris, S.B. Ross-Murphy, *Carbohydr. Res.*, 178 (1988) 93-110.
- 364 [3] J. Dekker, J.W.A. Rossen, H.A. Büller, A.W.C. Einerhand, *Trends Biochem. Sci.*, 27 (2002)  
365 126-131.
- 366 [4] T. Yu, G.P. Andrews, D.S. Jones, *Mucoadhesion and characterization of mucoadhesive*  
367 *properties*, in: *Mucosal Delivery of Biopharmaceuticals: Biology, Challenges and Strategies*,  
368 Springer US, 2014, pp. 35-58.
- 369 [5] I. Carlstedt, J.K. Sheehan, A.P. Corfield, J.T. Gallagher, *Essays in Biochemistry*, 20 (1985) 40-  
370 76.
- 371 [6] T. Ichikawa, K. Ishihar, *Protective Effects of Gastric Mucus*, in: P. Tonino (Ed.) *Gastritis and*  
372 *Gastric Cancer - New Insights in Gastroprotection, Diagnosis and Treatments*, InTech, 2011.
- 373 [7] R. Bansil, B.S. Turner, *Current Opinion in Colloid and Interface Science*, 11 (2006) 164-170.
- 374 [8] A.S.R. Donald, *BBA - Protein Structure*, 317 (1973) 420-436.
- 375 [9] M. Scawen, A. Allen, *Biochemical Journal*, 163 (1977) 363-368.
- 376 [10] S.E. Harding, G.G. Adams, F. Almutairi, Q. Alzahrani, T. Erten, M. Samil Kök, R.B. Gillis, in:  
377 *Methods in Enzymology*, Academic Press Inc., 2015, pp. 391-439.
- 378 [11] R.B. Gillis, G.G. Adams, B. Wolf, M. Berry, T.M.D. Besong, A. Corfield, S.M. Kök, R.  
379 Sidebottom, D. Lafond, A.J. Rowe, S.E. Harding, *Carbohydrate Polymers*, 93 (2013) 178-183.
- 380 [12] K. Jumel, F.J. Fogg, D.A. Hutton, J.P. Pearson, A. Allen, S.E. Harding, *European biophysics*  
381 *journal : EBJ*, 25 (1997) 477-480.
- 382 [13] K. Jumel, I. Fiebrig, S.E. Harding, *International Journal of Biological Macromolecules*, 18  
383 (1996) 133-139.
- 384 [14] J.K. Sheehan, I. Carlstedt, *Models for the macromolecular structure of mucus glycoproteins*,  
385 in: S.E. Harding, A.J. Rowe (Eds.) *Dynamic Properties of Biomolecular Assemblies*, Royal Society  
386 of Chemistry, Cambridge, UK, 1989, pp. 256-275.



- 387 [15] J.K. Sheehan, S. Kirkham, M. Howard, P. Woodman, S. Kutay, C. Brazeau, J. Buckley, D.J.  
388 Thornton, *J. Biol. Chem.*, 279 (2004) 15698-15705.
- 389 [16] C.M. Caramella, S. Rossi, F. Ferrari, M.C. Bonferoni, G. Sandri, *Adv. Drug Deliv. Rev.*, 92  
390 (2015) 39-52.
- 391 [17] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, *Analytical Chemistry*, 28  
392 (1956) 350-356.
- 393 [18] V. Hoang, M. Williams, H. Simpson, *Veterinary parasitology*, 170 (2010) 253-261.
- 394 [19] S.E. Harding, *Progress in Biophysics and Molecular Biology*, 68 (1997) 207-262.
- 395 [20] M.L. Huggins, *Journal of the American Chemical Society*, 64 (1942) 2716-2718.
- 396 [21] E.O. Kraemer, *Ind. Eng. Chem.*, 30 (1938) 1200-1203.
- 397 [22] S. Ohara, K. Ishihara, K. Hotta, *Comparative Biochemistry and Physiology -- Part B:*  
398 *Biochemistry and*, 106 (1993) 153-158.
- 399 [23] M.P. Deacon, S.S. Davis, J.H. Waite, S.E. Harding, *Biochemistry*, 37 (1998) 14108-14112.
- 400 [24] J.K. Sheehan, I. Carlstedt, *Biochemical Journal*, 217 (1984) 93-101.
- 401 [25] J. Argenis Caicedo, J.E. Perilla, *Ingen. Invest.*, 35 (2015) 43-48.
- 402 [26] C.D. Hurd, *Journal of Chemical Education*, 47 (1970) 481-482.
- 403 [27] F.J.J. Fogg, D.A. Hutton, K. Jumel, J.P. Pearson, S.E. Harding, A. Allen, *Biochemical Journal*,  
404 316 (1996) 937-942.
- 405 [28] S.E. Harding, A.S. Abdelhameed, G.A. Morris, *Polym. Int.*, 60 (2011) 2-8.
- 406 [29] G.A. Morris, G.G. Adams, S.E. Harding, *Food Hydrocolloids*, 42 (2014) 318-334.
- 407 [30] F. Tanaka, *Polymer Physics: Applications to Molecular Association and Thermoreversible*  
408 *Gelation*, (2011).
- 409 [31] O. Svensson, (2008). *Interactions of Mucins with Biopolymers and Drug Delivery Particles.*  
410 *PhD Thesis, University of Malmo.*

- 411 [32] K. Tømmeraas, P.O. Wahlund, *Carbohydrate Polymers*, 77 (2009) 194-200.
- 412 [33] E.R. Morris, A.N. Cutler, S.B. Ross-Murphy, D.A. Rees, J. Price, *Carbohydrate Polymers*, 1  
413 (1981) 5-21.
- 414 [34] M.R. Kasaai, *Journal of Applied Polymer Science*, 100 (2006) 4325-4332.
- 415 [35] G.A. Morris, T.J. Foster, S.E. Harding, *Carbohydrate Polymers*, 48 (2002) 361-367.
- 416 [36] P. Georgiades, E. Di Cola, R.K. Heenan, P.D.A. Pudney, D.J. Thornton, T.A. Waigh,  
417 *Biopolymers*, 101 (2014) 1154-1164.
- 418 [37] J.P. Celli, B.S. Turner, N.H. Afdhal, R.H. Ewoldt, G.H. McKinley, R. Bansil, S. Erramilli, S.  
419 Erramilli, *Biomacromolecules*, 8 (2007) 1580-1586.
- 420