Carbohydrate Polymers 131 (2015) 142–151

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Alginate as a protease inhibitor in vitro and in a model gut system; selective inhibition of pepsin but not trypsin

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ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form 14 May 2015
Accepted 25 May 2015
Available online 4 June 2015

Keywords:
Alginate
Pepsin
Trypsin
Proteolysis
Model gut system

ABSTRACT

Alginates are widely used in the food and medical industries, including as a Gastro-Oesophagul Reflux treatment. This work investigates the inhibitory effects of alginate on the reflux aggressors trypsin and pepsin and the role of alginate-substrate binding, pH and alginate structure on inhibition. Alginates were shown to reduce pepsin activity by up to 53.9% (±9.5SD) in vitro. Strong positive correlation between alginate mannuronate residue frequency and levels of pepsin inhibition was observed. Limited inhibition of trypsin was shown. Viscometric observations of pH dependent interactions between alginate and protein suggest a mechanism whereby pH dependent ionic interactions reduce substrate availability to enzyme at acidic pH. To understand how dietary protein digestion is affected by alginate, proteolytic digestion was investigated in an in vitro model of the upper digestive tract. Significant inhibition of proteolysis was shown in the gastric phase of digestion, but not the small intestinal phase.

1. Introduction

Alginates have previously been shown to inhibit pepsin activity *in vitro*, Sunderland, Dettmar, and Pearson (2000) showed alginates could inhibit pepsin activity by 52% in vitro. Strugala, Kennington, Campbell, Skjak-Braek, and Dettmar (2005), showed significant correlations between alginate structure and levels of inhibition, with high frequency of mannuronic acid residues (F[M]) alginates tending to inhibit better than those high in guluronic acid residues (F[G]). However the mechanism of pepsin inhibition is poorly understood, as is how alginate effects dietary proteolysis and the activity of small-intestinal proteases such as trypsin.

Pepsin has been shown to be a major aggressor in GORD (Gastro-Oesophagul Reflux disease) (Strugala, Avis, Jolliffe, Johnstone, & Dettmar, 2009) with *in vivo* addition of pepsin to the oesophagus resulting in reflux-like oesophagitis in animal models (Goldberg, Dodds, Gee, Montgomery, & Zboralske, 1969). Alginates are used in the treatment of reflux (Sunderland et al., 2000). The primary mechanism of alginate treatment of reflux is the formation of an alginate raft upon coming into contact with stomach acid; sodium or potassium bicarbonate in the formulation releases carbon dioxide which becomes trapped in the gel as a foam, allowing the alginate acid-gel raft to float on the top of the stomach contents and create a physical barrier to refluxate (Dettmar, Sykes, Little, & Bryan, 2006; Sengupta, Shah, & Shah, 2015). The inhibitory action of alginate on pepsin is thought to be a secondary mechanism for the anti-reflux activity of alginate based agents (Strugala et al., 2009).

The damaging potential of the pancreatic protease trypsin has also been demonstrated in gastroduodenal refluxate. With the use of proton pump inhibitors in the treatment of reflux disease, gastric pH becomes elevated. During a gastro-duodenal reflux event, if trypsin passes through the stomach at pH 4.0 or above (or rapidly through at low pH of 2 or less) it can retain proteolytic activity and cause damage (Pearson et al., 2011). We therefore seek to investigate the effects of alginate on trypsin activity and examine a potential role of alginate in the management of trypsin mediated damage in gastro-duodenal reflux.

Furthermore, alginates are widely used in the food and medical industries and have a range of bioactive properties, as reviewed elsewhere (Brownlee et al., 2005). Alginates have been shown to be potent inhibitors of pancreatic lipase activity and are being investigated as a potential tool for the management of obesity (Balasubramaniam et al., 2013). It is therefore important to understand the secondary effects alginate may have on protein digestion.

In this work we therefore seek to investigate the inhibitory action of alginate on trypsin and pepsin *in vitro* and to characterise the effects of varying alginate structure on any inhibition observed. We also investigate the pH dependency of viscometric

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http://dx.doi.org/10.1016/j.carbpol.2015.05.062

interactions between alginate and protein substrates. Finally the inhibitory effects of alginate on protein digestion are investigated in a model gut system.

2. Methods

2.1. Materials

All alginate samples tested were kindly supplied by FMC Biopolymer and Technostics Ltd (Hull, UK). Bovine serum albumin (BSA) was purchased from VWR Jencons. Unless otherwise stated, all other chemicals and reagents were purchased from Sigma–Aldrich (Poole, UK). The structures of the alginate samples were characterised by ^{13}C NMR neighbour diad analysis and the full characteristics of all samples are shown in Table 1.

2.2. N-Terminal proteolytic assay

Proteolytic activity was determined using an adapted version of the N-terminal assay developed by Lin, Means, and Feeney (1969) and modified by Hutton, Allen, Pearson, Ward, and Venables (1986). The activity assay was scaled down to a 96-well microplate as reported in Ali, Parikh, Chater and Pearson (2013). Alginate was prepared in 0.05 M phosphate (pH 2.5) as alginate was shown to alter the pH of the reaction mixture when made up in distilled water as per Strugala et al. (2005).

For pepsin activity, 10 μg/ml pepsin (prepared 10 min prior to assay) and 10 mg/ml succinyl albumin were each made up in 0.05 M phosphate buffer at pH 2.5. In order to prevent precipitation, alginate samples were prepared at 10 mg/ml in the basic side of the buffer and then diluted at a 1:1 ratio with the acidic side of the buffer to give a concentration of 5 mg/ml in 0.05 M phosphate buffer. Trinitrobenzosulfonic acid (TNBS) was prepared at 2 μl/ml in deionised water. Sodium bicarbonate was prepared 10% w/v. Pentosan polysulphate (SP54) at 5 mg/ml was used as a positive inhibition control (Bianchi & Cook, 1964; Cook & Drill, 1967).

For trypsin activity assay, 5 μg/ml trypsin (prepared 10 min prior to assay) and 10 mg/ml succinyl albumin were each made up in 0.066 mM Sorensen's phosphate buffer at pH 7. Alginate samples were prepared at 5 mg/ml in 0.066 M Sorensen's phosphate buffer. Trinitrobenzosulfonic acid (TNBS) and sodium bicarbonate were prepared as above. Soybean trypsin inhibitor was used as a positive inhibition control.

Eighteen alginates were tested for their ability to inhibit pepsin and trypsin activity. All alginate samples were tested at three concentrations; 5, 2.5 and 1.25 mg/ml. This gave concentrations in the reaction mixture of 1.36, 0.68 and 0.34 mg/ml, respectively.

Thirty microlitres of alginate sample was pre-incubated with 50 μl succinyl albumin substrate for 60 min on a shaker. At T0 30 μl enzyme solution or buffer blank was added as appropriate and the plate was incubated for 30 min at 37 °C. After 30 min, 50 μl sodium bicarbonate and 50 μl TNBS was added, mixed and the plate was incubated for 15 min at 55 °C. At T45, 50 μl SDS (10% w/v) and 50 μl 1 M hydrochloric acid were added and the plate was left to stand until all wells had stopped effervescing, and absorbance was measured at 340 nm using a Biotek 96 well microplate reader (Elx808 Biotek, Bedfordshire, UK).

To calculate percentage pepsin inhibition the following formula was used:

$$\text{Precentage pepsin inhibition} = \frac{\text{polymer sample} - \text{sample control}}{\text{pepsin control} - \text{background control}} \times 100$$

Table 1
Codes and molecular characteristics for alginates used in this study presented alongside levels of pepsin and trypsin inhibition. F(G) is the fraction of the alginate polymer composed of guluronate and F(M) the fraction of mannuronate. $n(G>1)$ is the number of consecutive guluronate residues above 1.

	F(G)	F(M)	F(GG)	F(MM)	F(MGM)	F(GGG)	$n(G>1)$	Pepsin inhibition			Trypsin inhibition		
								5 mg/ml	2.5 mg/ml	1.25 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml
FMC 2	0.66	0.34	0.54	0.23	0.076	0.51	15.47	26.00 ± 8.78	19.99 ± 7.46	−3.07 ± 13.75	11.46 ± 6.54	2.74 ± 2.86	1.53 ± 3.02
FMC 3	0.68	0.32	0.57	0.21	0.069	0.53	14.66	11.38 ± 10.63	3.95 ± 13.84	−2.43 ± 8.87	4.39 ± 3.12	7.83 ± 1.48	5.88 ± 6.64
FMC 4	0.51	0.49	0.34	0.32	0.124	0.3	8.97	25.46 ± 8.69	17.80 ± 6.19	10.96 ± 5.67	4.92 ± 6.55	7.68 ± 2.55	3.52 ± 6.13
FMC 5	0.53	0.47	0.36	0.31	0.123	0.32	9.67	33.95 ± 5.25	25.20 ± 6.92	12.78 ± 8.99	10.44 ± 1.99	7.45 ± 2.59	7.87 ± 2.68
FMC 6	0.52	0.48	0.35	0.31	0.122	0.3	8.15	19.23 ± 7.81	13.06 ± 9.93	1.91 ± 9.78	3.85 ± 3.41	8.25 ± 5.77	3.54 ± 3.87
FMC 7	0.42	0.58	0.24	0.4	0.133	0.2	6.47	29.43 ± 5.44	16.89 ± 6.22	11.78 ± 6.48	7.29 ± 2.91	5.60 ± 0.79	−1.67 ± 2.49
FMC 9	0.45	0.55	0.26	0.36	0.136	0.2	5.78	42.57 ± 5.25	24.73 ± 8.84	14.54 ± 10.16	−10.31 ± 10.99	−10.02 ± 25.23	−4.31 ± 12.13
FMC 10	0.42	0.58	0.21	0.37	0.14	0.14	3.96	39.41 ± 6.71	13.38 ± 14.73	2.52 ± 11.24	−1.70 ± 12.13	5.37 ± 5.24	−1.13 ± 9.47
FMC 12	0.35	0.65	0.19	0.49	0.111	0.13	4.54	38.13 ± 4.52	20.59 ± 10.28	11.79 ± 6.57	−2.83 ± 11.61	0.35 ± 9.64	4.97 ± 17.73
FMC 13	0.34	0.66	0.17	0.49	0.124	0.12	4.63	45.99 ± 5.97	31.42 ± 10.03	13.63 ± 4.92	4.23 ± 8.16	2.59 ± 13.33	2.72 ± 11.59
LF120	0.424	0.576	0.24	0.391	0.156	0.183	4.7	44.73 ± 10.98	28.46 ± 10.68	1.21 ± 7.74	6.22 ± 7.71	3.79 ± 10.77	3.50 ± 6.66
LFR560	0.633	0.367	0.505	0.239	0.096	0.45	9.9	32.42 ± 5.31	18.34 ± 6.49	9.94 ± 7.85	−6.76 ± 11.41	0.77 ± 10.58	0.25 ± 8.08
LF10L	0.45	0.553	0.257	0.362	0.153	0.19	4.4	42.66 ± 6.23	22.84 ± 5.84	8.18 ± 7.93	−5.86 ± 8.66	−4.38 ± 7.84	−9.05 ± 6.65
H120L	0.45	0.551	0.276	0.379	0.15	0.22	5.9	46.09 ± 9.53	24.37 ± 11.90	9.81 ± 6.54	3.07 ± 5.70	4.66 ± 2.60	2.53 ± 5.56
SF120	0.664	0.336	0.545	0.218	0.083	0.484	9.6	21.93 ± 5.42	8.83 ± 5.56	0.45 ± 5.31	−7.20 ± 7.96	2.23 ± 4.00	10.05 ± 5.19
SF200	0.68	0.322	0.573	0.218	0.079	0.537	16.7	13.10 ± 6.03	15.93 ± 6.37	14.11 ± 13.58	−0.89 ± 2.46	−1.35 ± 3.10	−5.52 ± 2.53
SF/LF	0.66	0.336	0.548	0.22	0.081	0.506	13.8	15.56 ± 5.40	4.08 ± 6.86	−5.26 ± 6.79	0.90 ± 7.99	−2.88 ± 10.97	−1.73 ± 2.90
SF60L	0.411	0.589	0.219	0.393	0.155	0.133	3.3	43.87 ± 7.36	15.50 ± 8.88	4.12 ± 11.67	−2.11 ± 14.83	−2.05 ± 17.15	−9.14 ± 23.66