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Inhibition of pepsin activity by alginates in vitro and the effect of epimerization

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Abstract

Alginates are versatile biopolymers used extensively in the food, textile and pharmaceutical industries. One of the major uses is in the treatment of reflux disease and here we investigate whether alginates can influence pepsin activity, a major aggressor in reflux disease. The primary uronic acid structure of alginates can be altered using epimerase technology and we test tailor-made alginates to identify the optimal structure for pepsin inhibition. Pepsin activity in the presence of alginates was studied using an in vitro N-terminal assay and enzyme kinetics using a chromagenic peptide. The data described showed clearly that alginates were capable of concentration dependently reducing the activity of pepsin in a non-competitive manner, in vitro. This was variable between different alginates of wide ranging structure and size with positive correlation with alternating sequences of mannuronic and guluronic acid. We hypothesize that alginates may have a more extensive role in the treatment of reflux disease by inhibiting pepsin, a damaging component of the refluxate.
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Keywords: Alginate; Pepsin; Gastroesophageal reflux; Epimerase; Enzyme kinetics

1. Introduction

Alginates are polysaccharides that provide the main structural component of brown algae (*Phaeophycae*) such as *Laminaria hyperborea*, *Lessonia nigrescens* and *Ascophyllum nodosum*. They are also produced as an exopolysaccharide by some bacteria such as *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. Alginates are linear copolymers of (1–4) linked β-D-

Abbreviations: M, mannuronic acid; G, guluronic acid; GERD, gastro-esophageal reflux disease; ^{1}H NMR, nuclear magnetic resonance; *F*, molar fraction; SEC-MALLS, size exclusion chromatography-multi angle laser light scattering; TNBS, trinitrobenzenesulphonic acid; [*S*], substrate concentration; *V*, initial reaction velocity; V_{max}, maximum enzyme velocity; K_m, Michaelis–Menton constant; *r*, Pearson's correlation coefficient

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mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G). The M and G residues can exist in homopolymeric regions (M block or G block) or heteropolymeric regions (MG block) (Smidsrod and Draget, 1996).

Alginates are among the most versatile biopolymers with respect to their uses in the food, textile and pharmaceutical industries (Onsoyen, 1996). They function as thickeners, stabilizers, gel-forming and film-forming agents. Within the pharmaceutical industry, applications include controlled release mediators, dental impression materials, wound dressings, anti-reflux medicines (Onsoyen, 1996) and microencapsulation (Skjak-Braek and Espevik, 1996). The many versatile applications of alginates are a function of their structure as different sequences of M and G infer different physical and chemical properties (Smidsrod and Draget, 1996).

It is now possible to control the primary structure of alginates and thereby the physical and chemical properties. The processes, genes and enzymes that control the structure of alginate produced by the bacteria *P. aeruginosa* and *A. vinelandii* are now well understood. The bacteria initially synthesize an alginate consisting solely of M residues (polymannuronate) and secrete it into the extracellular space. From this point the conversion of M into G within the polymer chain is catalyzed by mannuronan C-5 epimerase enzymes (May and Chakrabaty, 1994). The genes that encode the mannuronan C-5 epimerases have been isolated in both *P. aeruginosa* and *A. vinelandii* and designated $AlgE_1$–$AlgE_7$ each producing an enzyme with a different pattern of activity (Ertesvag et al., 1994; Svanem et al., 1999). They can then be used to modify the sequence of an alginate, either of seaweed or bacterial origin, in vitro (Ertesvag et al., 1994; Ertesvag et al., 1995). This enables the properties of alginates to be controlled and tailored to specific commercial applications.

Alginates have been demonstrated to possess several biological activities. Certain alginates, particularly those with high M content, have immune stimulating effects such as cytokine induction (Flo et al., 2000; Jahr et al., 1997) and protection against pathogenic invasion (Skjak-Braek and Espevik, 1996). Alginates exhibit bioadhesive ability to oesophageal tissue (Batchelor et al., 2002; Richardson et al., 2004, 2005) and can positively interact with mucin (Batchelor et al., 2000; Taylor et al., 2005). In preliminary studies, presented as meeting abstracts, alginates have been shown to upregulate the process of cell migration and so potentially speed up the repair of wounds (Dunne et al., 2002), protect rat gastric mucosa from stress and indomethacin induced ulcers (Del Buono et al., 2001). The process of fluid phase endocytosis is also upregulated in the presence of alginates (McPherson et al., 2002), they have the ability to reduce the activity of pepsin (Sunderland et al., 2000), and have intracellular molecular effects (Johnston et al., 2002; Dettmar et al., 2004).

Many of these bioactive properties have relevance to the treatment and/or prevention of gastro-esophageal reflux disease (GERD). This is the retrograde movement of the gastric contents into the esophagus resulting in symptoms of heartburn. Pepsin is a major aggressive factor in the gastric refluxate and is responsible for much of the esophageal damage seen in GERD sufferers (Salo et al., 1983; Tobey et al., 2001). Alginate containing products are currently used in the treatment of GERD (e.g. Gaviscon Advance®) although primarily as a physical barrier to prevent reflux. However, it is apparent that alginates may have greater potential for use in the treatment of GERD. Here we utilize epimerase technology to design new alginates and evaluate them for their ability to inhibit pepsin activity and to determine the alginate structure required for optimal activity in vitro with a long term view to pharmaceutical product development.

2. Materials and methods

2.1. Alginates

Current commercially available seaweed alginates were supplied by FMC Biopolymer, Drammen, Norway. Novel alginates of bacterial (mannuronan) and seaweed origin treated with the mannuronan C-5 epimerases $AlgE_1$, $AlgE_4$ and $AlgE_6$ were supplied by Gudmund Skjåk-Bræk, NOBIPOL, Trondheim, Norway. Alginates were characterized by ^1H NMR to resolve the fraction of monad, diad and triad frequencies (F_x) (Smidsrod and Draget, 1996), intrinsic viscosity and SEC-MALLS to determine molecular weight. Table 1 shows the 39 different alginates used in this

Table 1
Details of the alginates used in this study

	Source	Epi	F_G	F_M	F_{GG}	F_{MG}	F_{MM}	F_{MGG}	F_{MGM}	F_{GGG}	$N_{G>1}$	MWt	η
A	Mannuronan	None	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0	584.4	2457
B	Mannuronan	None	0.00	1.00	0.00	0.00	1.00	0.00	0.00	0.00	0		860
C	Mannuronan	$AlgE_1$	0.44	0.56	0.31	0.12	0.44	0.04	0.10	0.27	8	442.4	1719
D	Mannuronan	$AlgE_1$	0.52	0.48	0.38	0.14	0.34	0.04	0.12	0.34	11	436.8	1629
E	Mannuronan	$AlgE_1$	0.68	0.32	0.59	0.09	0.23	0.03	0.08	0.56	22	374.8	1698
F	Mannuronan	$AlgE_1$	0.80	0.20	0.70	0.10	0.10	0.02	0.09	0.68	44	423.8	1731
G	Mannuronan	$AlgE_4$	0.47	0.53	0.00	0.47	0.06	0.00	0.47	0.00	0	456.7	1110
H	Mannuronan	$AlgE_4$	0.47	0.53	0.00	0.47	0.06	0.00	0.47	0.00	0	440.1	912
I	Mannuronan	$AlgE_4$	0.47	0.53	0.00	0.47	0.06	0.00	0.47	0.00	0	95.7	1116
J	Mannuronan	$AlgE_4$	0.47	0.53	0.00	0.47	0.06	0.00	0.47	0.00	0	56.7	1537
K	Mannuronan	$AlgE_4$	0.47	0.53	0.00	0.47	0.06	0.00	0.47	0.00	0	27.8	949
L	Mannuronan	$AlgE_4$	0.17	0.83	0.00	0.17	0.66	0.00	0.17	0.00	0	438.7	1004
M	Mannuronan	$AlgE_4$	0.36	0.64	0.00	0.36	0.28	0.00	0.36	0.00	0	426.9	1110
N	Mannuronan	$AlgE_4$	0.46	0.54	0.00	0.46	0.08	0.00	0.46	0.00	0	367.3	1019
O	D. pot	None	0.32	0.68	0.20	0.12	0.56	0.05	0.07	0.16	6	241.1	1106
P	D. pot	$AlgE_1$	0.70	0.30	0.55	0.15	0.15	0.08	0.09	0.47	7	262.4	1170
Q	D. pot	$AlgE_1$	0.62	0.38	0.44	0.18	0.20	0.07	0.12	0.37	8	211.8	
R	D. pot	$AlgE_4$	0.54	0.46	0.28	0.26	0.20	0.08	0.21	0.19	4	194.8	
S	D. pot	$AlgE_6$	0.62	0.38	0.45	0.17	0.21	0.12	0.09	0.34	5	202.0	
T	L. hyp leaf	None	0.49	0.51	0.33	0.16	0.35	0.04	0.12	0.29	9	260.6	
U	L. hyp leaf	$AlgE_1$	0.68	0.32	0.53	0.15	0.16	0.06	0.12	0.47	9	251.2	
V	L. hyp leaf	$AlgE_6$	0.67	0.33	0.52	0.15	0.18	0.06	0.08	0.46	9	226.6	
W	L. hyp leaf	None	0.51	0.49	0.34	0.17	0.31	0.06	0.14	0.28	7	41.0	
X	L. hyp leaf	$AlgE_1$	0.71	0.29	0.54	0.17	0.13	0.06	0.12	0.48	9		
Y	L. hyp leaf	$AlgE_4$	0.66	0.34	0.43	0.23	0.11	0.07	0.18	0.36	7		
Z	L. hyp stem	None	0.63	0.37	0.51	0.13	0.24	0.05	0.10	0.45	9.9	34.7	1.69
AA	L. hyp stem	None	0.66	0.34	0.55	0.12	0.22	0.06	0.08	0.48	9.6	195.0	8.41
AB	L. hyp stem	None	0.66	0.34	0.55	0.12	0.22	0.04	0.08	0.51	13.8	295.0	19.82
AC	L. hyp stem	None	0.68	0.32	0.57	0.11	0.22	0.04	0.08	0.54	16.7	387.0	15
AD	L. nig	None	0.45	0.55	0.26	0.19	0.36	0.07	0.15	0.19	4.4	75.0	
AE	L. nig	None	0.42	0.58	0.24	0.19	0.39	0.06	0.16	0.18	4.7	221.0	7.97
AF	L. nig	None	0.41	0.60	0.22	0.20	0.39	0.08	0.16	0.14	3.3	325.0	11.98
AG	L. nig	None	0.45	0.55	0.28	0.17	0.38	0.05	0.15	0.22	5.9	397.0	14.6
AH	L. nig	None	0.40	0.60	0.21	0.41	0.19	0.14	0.06	0.16	4	696.0	
AI	L. nig	None	0.42	0.58	0.24	0.39	0.18	0.18	0.06	0.15	5	411.0	
AJ	L. nig	None	0.45	0.55	0.26	0.37	0.19	0.20	0.06	0.15	5	293.0	
AK	L. nig	None	0.48	0.52	0.30	0.34	0.18	0.24	0.07	0.14	5	176.0	
AL	L. nig	None	0.52	0.48	0.35	0.31	0.17	0.28	0.07	0.13	5	95.0	
AM	L. nig	None										391.0	

D. pot, *Durvillea potatorum*; L. hyp, *Laminaria hyperborea*; L. nig, *Lessonia nigrescens*; Epi, epimerase; MWt, molecular weight in KDa. F, molar fraction of sequence in subscript; G, guluronic acid; M, mannuronic acid; $N_{G>1}$, average length of G block; η, intrinsic viscosity (g/ml).

study. Alginate solutions were prepared by adding alginate powder to deionized water stirred using an overhead stirrer.

2.2. Other materials

Porcine pepsin A EC.3.4.23.1 (2870 units/mg) and Pepstatin A were obtained from Sigma-Aldrich. Trinitrobenzenesulphonic acid (TNBS) was obtained from Fluka. All other reagents were obtained from Fisher Scientific UK and were of AnalaR grade.

2.3. N-terminal assay

Proteolytic activity was determined using the N-terminal assay method (Hutton et al., 1986), which is a sensitive colorimetric method to detect newly formed N-terminals from digested protein substrates.

This assay was performed at pH 2.2, where pepsin is at optimal activity without risking autodigestion, as would occur at lower pH's, and also represents a pH were the protein substrate is soluble. Porcine pepsin was diluted in 0.01 M HCl pH 2.2 to a concentration of 20 μg/ml and a standard curve produced ranging from 0 μg to 2 μg pepsin (total volume 200 μl). For test solutions 100 μl of 5 mg/ml aqueous alginate solution was added to 100 μl pepsin standard. Hundred microliters of 5 μg/ml pepstatin A in 0.01 M HCl pH 2.2 was used as a positive control. Five hundred microliters of 10 mg/ml succinyl albumin substrate in 0.01 M HCl, pH 2.2 was added to the enzyme solutions and incubated at 37 °C for 30 min. The reaction was halted by addition of 500 μl 4% $NaHCO_3$. Color was developed with 500 μl 10 mM trinitrobenzenesulphonic acid (TNBS) and incubated at 50 °C for 10 min. After addition of 500 μl 10% sodium dodecyl sulphate and 250 μl 1 M HCl the absorbance was read at 340 nm against reagent blank. The mean percentage inhibition of pepsin activity was determined from the standard curve.

This assay is linear between 0 μg and 3 μg porcine pepsin. The limit of detection of the assay is 0.05 μg porcine pepsin, which is equivalent to 95% inhibition of 1 μg pepsin and 97.5% inhibition of 2 μg pepsin. The coefficient of variation in the range tested is 4%.

pH of the reaction was not significantly affected by addition of aqueous alginate to the exclusion of 0.01 M HCl. Mean pH of pepsin standard curve was 2.2 compared to 2.3 in the presence of alginate, a difference that has little influence on pepsin activity.

2.4. Viscosity

To provide additional characterization of the alginates used in this study the viscosity of alginate solutions was measured using a Bohlin CVO controlled stress rheometer (Bohlin, Cirencester, Gloucestershire, UK) with small sample adapter cup and bob geometry with gap size 7 mm. Equilibrium flow curves were obtained at 25 °C with applied shear stress increasing stepwise from 1 Pa to 100 Pa.

2.5. Kinetic assay

Enzyme kinetics of pepsin activity were determined using the established method of Dunn (Dunn et al., 1984). Chromagenic heptapeptide (Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu) was dissolved in 0.1 M sodium formate buffer, pH 3 (substrate concentration [S] 0–110 nM). Reactions were performed at 37 °C and absorbance measured at 300 nm over 1 min using a quartz cuvette with 1 cm pathlength. To 800 μl substrate was added 25 μl porcine pepsin in 0.1 M sodium formate buffer (final concentration 17.1 nM) and 25 μl of either buffer alone, pepstatin A in 0.1M sodium formate buffer (final concentration 8.1 nM, 20.2 nM and 100.9 nM) or alginate in water (final concentration 3.8 μM and 7.3 μM). [S] versus initial reaction velocity (V) was plotted and V_{max} and K_m determined by applying Michaelis–Menton non-linear regression. The Lineweaver–Burke plot (1/[S] versus 1/V) was plotted to visualize inhibition parameters.

2.6. Statistical analysis

Data are expressed as mean (standard deviation). Pearson's correlation coefficient (r) was used to determine the relationship between inhibition and alginate characteristics. Data was considered significant if $p < 0.05$.

3. Results

3.1. Concentration-response effect

Eight alginates were tested for their ability to inhibit pepsin activity at varying concentrations. These alginates were chosen to represent both bacterial and seaweed sources, varying primary structure and molecular weight. Initial alginate concentrations (w/v) were 5 mg/ml, 2 mg/ml, 1 mg/ml, 200 μg/ml, 20 μg/ml and 2 μg/ml in deionized water. The range of ratios of alginate: pepsin ranged from alginate being 500× in excess to pepsin being 10× in excess by weight. Fig. 1 shows the concentration-response profile for the distinct alginates. Concentration-dependent inhibition of porcine pepsin was observed by all alginates, although the magnitude varied. Even at the lowest alginate concentration inhibition of pepsin activity was seen (5–20%) and this increased in a concentration dependent manner. Significant differences between the alginates were evident at 5 mg/ml and 2 mg/ml but not at lower concentrations. It was decided that 5 mg/ml alginate concentration would

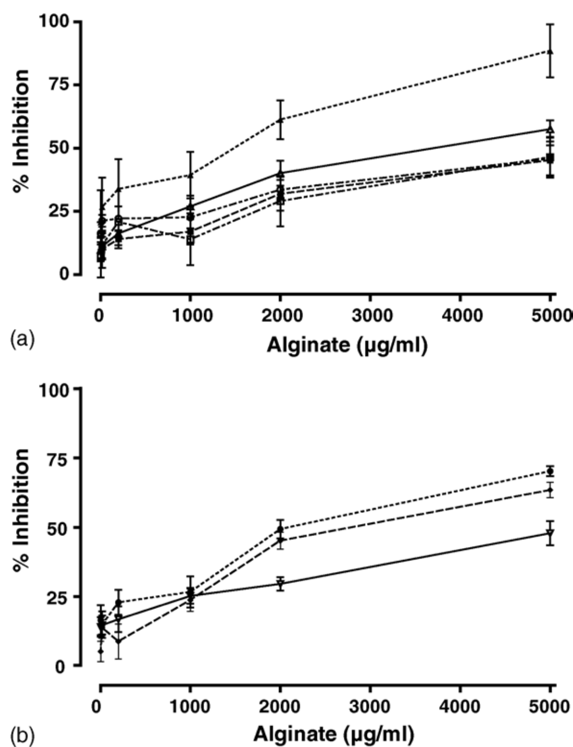

Fig. 1. Concentration-response curve of mean (S.E.) inhibition of 1 μg porcine pepsin by eight different alginates. All points represent a mean (S.E.) of five to seven replicates. (a) Bacterial derived alginates. (▲) G; (△) C; (◯) F; (■) A; (□) D. (b) Seaweed derived alginates (●) O; (♦) P; (▽) R.

be used in further experiments in order to differentiate between alginates.

3.2. Comparison of multiple alginates

A selection of 39 different alginates were tested for suppression of porcine pepsin activity at a concentration 5 mg/ml (w/v). These were from various seaweed and bacterial sources with and without epimerization to alter their primary structure which was determined by ^{1}H NMR (Table 1). Mean pepsin inhibition by the different alginates tested ranged from 39% to 81% for 1 μg pepsin ($n = 34$ alginates) and 38–75% for 2 μg pepsin ($n = 36$ alginates) (Table 2). There were two pools of alginates, a small subset that only produced approximately 40% inhibition and a larger subset that produced greater, but more variable, inhibition of pepsin activity. In all experiments pepstatin A produced complete abolition of pepsin activity.

3.3. Correlation

For each alginate the primary structural information was provided by ^{1}H NMR. Molecular weight was either calculated from intrinsic viscosity or measured using SEC-MALLS. In addition, actual viscosity of each alginate at 5 mg/ml was measured at 0.4 Pa applied shear stress (Table 2). These independent variables were then correlated against percentage inhibition of pepsin activity at 2 μg pepsin. Table 3 shows the Pearson's correlation coefficient (r) for each variable along with the significance of the relationship. No correlation was observed between variables related to size of the alginate molecule (molecular weight, intrinsic viscosity or viscosity at 0.4 Pa). There was a significant inverse relationship between G content of the alginate and pepsin inhibition. Those alginates with high F_G, F_{GG}, F_{GGG} and $N_{G>1}$, indicative of large areas of G block, had poor ability to inhibit pepsin activity. Conversely, there was a positive correlation between F_M and pepsin inhibition although long sequences of M had no association. Sequences of alternating monomers ($F_{GM/MG}$, F_{MGM}) did show a positive relationship with increasing proportion of alternating structure giving greater pepsin inhibition. Scatter profiles of significantly associated variables are shown in Fig. 2.

3.4. Enzyme kinetics

Pepsin digestion of the chromogenic peptide exhibited Michaelis–Menton kinetics reaching saturation as $[S]$ increased. Increasing concentrations of pepstatin A shifted the $[S]$ versus V curve downwards (Fig. 3). Analysis of the Lineweaver–Burke plot showed that the x-intercept ($-1/K_m$) remained constant indicative of non-competitive inhibition.

A representative alginate sample (AG), previously shown to be biologically active in other assays, also shifted the $[S]$ versus V curve downwards as concentration of alginate increased (Fig. 4). Analysis of the Lineweaver–Burke plot showed that the x-intercept ($-1/K_m$) remained constant indicative of non-competitive inhibition like pepstatin A.

4. Discussion

The data described here shows clearly that alginates are capable of reducing the activity of pepsin in vitro.

Table 2
Mean (S.D.) percentage inhibition of pepsin achieved by each alginate at 5 mg/ml and the number of replicates tested (*n*)

Alginate	Percentage inhibition of 1 μg pepsin			Percentage inhibition of 2 μg pepsin			Viscosity @ 0.4 Pa (mPa s)
	Mean	S.D.	*n*	Mean	S.D.	*n*	
A	58.87	14.3	19	57.52	17.0	21	2758
B	71.45	8.7	6	59.73	8.2	6	21.3
C	57.56	8.2	6	54.94	8.3	6	570.5
D	47.35	16.4	6	41.38	11.3	6	223.3
E	41.41	7.4	6	42.65	9.3	6	235.2
F	46.05	13.6	6	37.84	11.4	6	88
G	76.61	20.0	14	72.76	13.4	15	54.6
H	65.00	8.4	7	67.42	4.1	7	34.5
I	70.84	12.6	7	70.59	8.2	7	50.3
J	65.03	6.9	6	65.08	5.0	6	172.3
K	66.73	7.6	6	68.11	6.3	6	35.3
L	70.67	7.5	6	68.56	4.9	6	39.9
M	59.54	4.9	6	63.30	3.6	6	47
N	57.66	11.6	6	63.11	5.9	6	54.8
O	70.29	4.4	6	67.95	2.2	6	52.2
P	65.20	7.1	6	60.68	5.4	6	ND
Q	64.46	3.3	6	62.01	6.4	6	ND
R	47.84	10.7	6	43.98	8.6	6	ND
S	60.41	11.3	6	58.75	6.6	6	ND
T	61.21	5.0	6	57.23	4.8	6	ND
U	55.80	11.8	6	55.49	7.1	6	ND
V	47.79	11.4	6	52.91	8.7	6	ND
W	46.19	13.0	6	ND			4.1
X	39.16	13.8	6	ND			4.6
Y	44.64	12.6	6	ND			4.2
Z	42.21	16.1	6	56.81		1	3.1
AA	ND			46.58		1	27.9
AB	ND			52.52		1	71.7
AC	ND			44.35		1	109.6
AD	ND			60.07		1	5.2
AE	66.19		1	54.20	11.1	2	21.3
AF	ND			55.98		1	64.9
AG	69.93	12.5	10	59.41	4.3	4	146.7
AH	78.88	15.22	2	75.01	8.46	2	ND
AI	80.58	0.33	2	71.28	5.58	2	ND
AJ	66.44	16.67	2	64.71	6.26	2	ND
AK	73.26	9.86	2	69.07	5.69	2	ND
AL	73.14	15.04	2	70.36	6.14	2	ND
AM	73.62	18.33	2	73.24	3.38	2	ND

Viscosity of 5 mg/ml alginate at 0.4 Pa (mPa s), ND, not determined.

This capacity is variable between different alginates of wide ranging structure and size. Complete abolition of pepsin activity was not achieved in this study. The greatest mean inhibition observed was 88.5% by alginate G (Table 1) at 5 mg/ml initial concentration, which represented alginate being 500-fold in excess of pepsin by weight. This alginate was a large molecular weight poly-alternating alginate produced by epimerization of bacterial Mannuronan to completion with $AlgE_4$. The alginates were able to suppress pepsin activity in a concentration dependent manner and even when the pepsin was in excess of the alginate by weight inhibition was still observed.

From this data it is clear that the structure of the alginate is the most important feature for determining the effect on pepsin activity. There was no signifi-

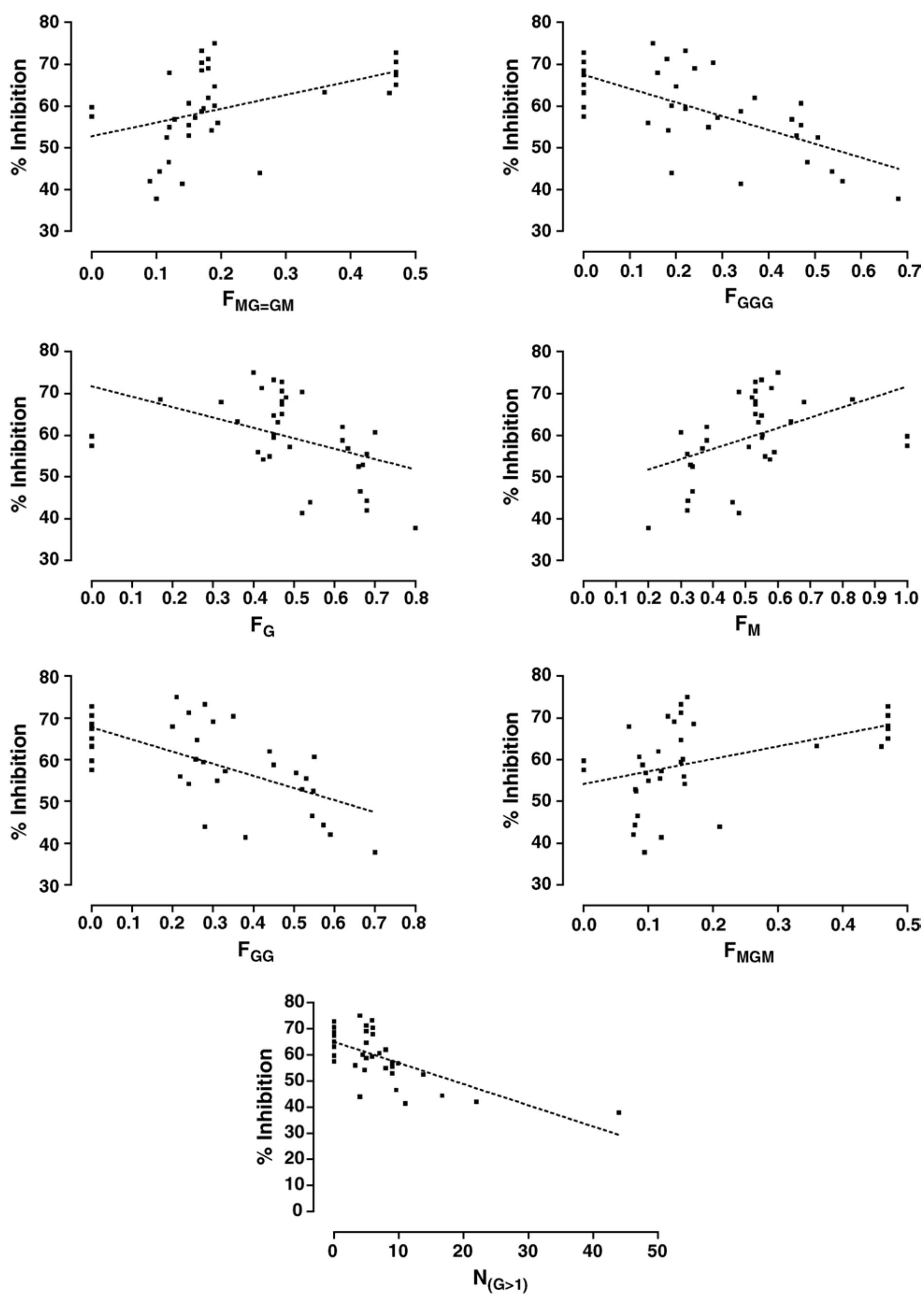

Fig. 2. Scatter plots of significantly correlated alginate characteristics against percent inhibition of 2 μg pepsin.

Table 3
Pearson r correlation coefficient and associated p value for percentage inhibition of 2 μg pepsin against independent variables of alginate characteristics

Parameter	*r*	*p*
Intrinsic viscosity	−0.062	0.7794
Viscosity at 0.4 Pa	0.118	0.5919
Molecular weight	−0.010	0.9551
F_G	−0.441	0.0071*
F_M	0.441	0.0072*
F_{GG}	−0.635	<0.0001*
$F_{GM/MG}$	0.449	0.0060*
F_{MM}	0.074	0.6663
$F_{MGG/GGM}$	−0.152	0.3763
F_{MGM}	0.439	0.0074*
F_{GGG}	−0.664	<0.0001*
$N_{G>1}$	−0.672	<0.0001*

* Correlation is considered significant if $p < 0.05$.

cant correlation between inhibitory activity and the molecular weight or viscosity of the alginates. Correlation with primary structure derived by ^{1}H NMR did provide an insight into the preferred structure. A significant negative correlation was observed for motifs representing G block in the alginate (F_G, F_{GG}, F_{GGG} and $N_{G>1}$). Lengths of G block infered rigidity and reduced acid solubility to the alginate and were associated with good ionic gel forming alginates (Smidsrod and Draget, 1996). In contrast, motifs representing alternating structure ($F_{GM/MG}$, F_{MGM}) were positively

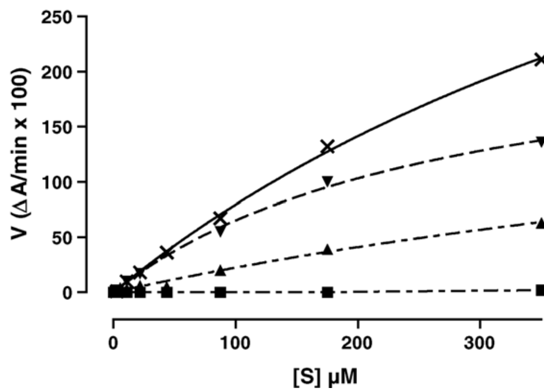

Fig. 3. Michaelis–Menton enzyme kinetics (substrate concentration [*S*], vs. initial velocity of reaction, *V*) of 84 nM porcine pepsin in 0.1 M sodium formate buffer, pH 3 on chromogenic substrate (Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu) in the presence of varying concentrations of pepstatin A in 0.1 M sodium formate buffer, pH 3 (× —— 0 nM; ▼ – – 8.1 nM; ▲ - - - 20.2 nM; ■ – - – 100.9 nM).

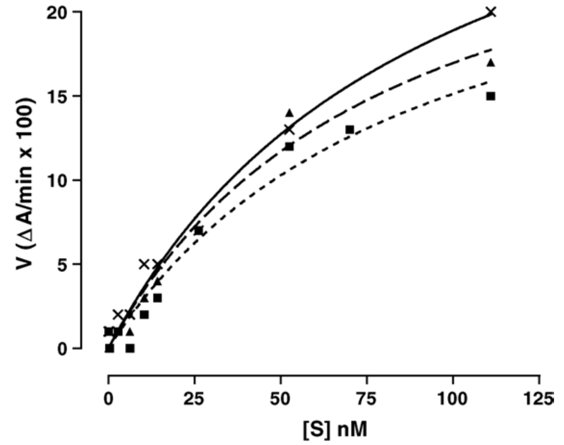

Fig. 4. Michaelis–Menton enzyme kinetics (substrate concentration [*S*], vs. initial velocity of reaction, *V*) of 17.1 nM porcine pepsin in 0.1 M sodium formate buffer, pH 3 on chromogenic substrate (Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu) in the presence of varying concentrations of alginate AG in deionized water (× —— 0 μM; ▲ – – 3.8 μM; ■ - - - 7.3 μM).

correlated with suppression of pepsin activity. Alternating sequences infered flexibility and good acid solubility on the alginate and were poor gel formers (Smidsrod and Draget, 1996). We suggest that either there may be a specific interaction of the pepsin active site with alternating sequences of alginate. Alternatively the physical properties of these alginates may favor a prolonged exposure to the enzyme namely due to their high acid solubility.

Michaelis–Menton enzyme kinetics demonstrated that alginates dose dependently inhibited porcine pepsin and that this was non-competitive. This was the same as seen for pepstatin A both in this study and in others (Roberts and Taylor, 2003), although pepstatin was far more effective.

This study also showed that suppression of pepsin activity by alginate occured with both large protein (succinyl albumin) and small peptide (chromogenic peptide) substrates using the two methods.

The clinical significance of the ability of alginates to reduce pepsin activity is their use in medications for the treatment of gastro-esophageal reflux disease. Alginate suspensions such as Liquid Gaviscon® and Gaviscon Advance® contain 50 mg/ml and 100 mg/ml alginate, respectively, concentrations far in excess of those tested in this study. The alginate contained in Gaviscon products (alginate Z in Table 1) gave

42% inhibition of pepsin at 5 mg/ml. Pepsin is a major aggressor to the delicate esophageal mucosa (Goldberg et al., 1969; Gotley et al., 1992; Lillemoe et al., 1982; Salo et al., 1983; Tobey et al., 2001) and laryngeal epithelium (Gill et al., 2002; Johnston et al., 2003; Koufman, 1991; Ludemann et al., 1998) during reflux. Therefore, any reduction in the proteolytic ability of this broad spectrum enzyme will reduce the damage done to these mucosal surfaces during a reflux event. Indeed, previous studies by our group have shown that the peptic activity of human gastric juice can also be suppressed by alginates and that the rank order of the alginates matches that seen with porcine pepsin (Sunderland, 2003).

Although it is believed that raising the pH of the refluxate using pharmacological acid suppression will negate the activity of pepsin this is a misnomer as pepsin can remain stable up to pH 7.8 depending on the isoform (Panetti et al., 2001; Piper and Fenton, 1965; Tasker, 2003). More importantly pepsin can retain a small amount of activity even at pH as high as 6.0 (Panetti et al., 2001; Piper and Fenton, 1965; Tasker, 2003). It is clear that pepsin will still retain activity and stability at and above the pH reached by the use of acid suppression drugs such as proton pump inhibitors and H_2 receptor antagonists (Panetti et al., 2001; Piper and Fenton, 1965; Tasker, 2003). This is particularly important if the pepsin molecule lingers and is exposed to a further episode of low pH. We speculate that alginate may have a direct action on pepsin and leave the acid unneutralized, which has little damaging effect on its own (Goldberg et al., 1969; Gotley et al., 1992; Lillemoe et al., 1982; Salo et al., 1983; Tobey et al., 2001). The potential side-effects of increasing pH and pharmacologically blocking acid secretion include bacterial overgrowth and increased *N*-nitroso compound formation (Moriya et al., 2002; Mowat et al., 2000), hypergastrinaemia and ECL cell hyperplasia (Waldum et al., 2004) and rebound acid secretion (Gillen and McColl, 2001; Gillen et al., 1999; Sandvik et al., 1997; Waldum et al., 1996). Therefore, the use of alginate containing products for the treatment of reflux in preference to systemic acid suppression, or in conjunction with, may be of benefit. One particular scenario where alginate therapy could be viable is in pregnancy when heartburn and regurgitation are common in the third trimester but systemic acid suppression drugs are contraindicated.

In summary, alginates have the ability to non-competitively reduce the proteolytic activity of pepsin in vitro in a variable but dose-dependent manner. Optimal pepsin inhibition is correlated to poly-alternating structure (MGMG) but inversely related to G block content and this can be controlled using epimerase technology. Alginates have considerable therapeutic potential in the treatment of esophageal and extra-esophageal reflux disease where pepsin is a major aggressor and warrants further investigation in man.

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