ELSEVIER

Biochimica et Biophysica Acta 1571 (2002) 55–63

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

The effect of guar galactomannan and water availability during hydrothermal processing on the hydrolysis of starch catalysed by pancreatic α-amylase

Suzanne L. Slaughter, Peter R. Ellis, Elizabeth C. Jackson, Peter J. Butterworth*

Biopolymers Group, Division of Life Sciences, King's College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NN, UK

Received 23 October 2001; received in revised form 11 March 2002; accepted 4 April 2002

Abstract

The effects of water-soluble nonstarch polysaccharides (sNSP) on human metabolism are considered to be beneficial because they decrease postprandial glycaemia and insulinaemia following ingestion of starch-rich foods. The mechanisms by which sNSP attenuate the postprandial rise in blood glucose are not well understood but their presence increases the viscosity of gastrointestinal contents, which affects physiological functions, e.g. gastric emptying and peristalsis. Increased viscosity and decreased water activity during hydrothermal treatment of starch could influence α-amylase action. Using guar galactomannan as a representative of sNSP, we found that galactomannan has a direct noncompetitive inhibitory effect on α-amylase with a K_i value of approximately 0.5% (3.3 μM). The inhibition is not time dependent and studies suggest direct binding of the enzyme to galactomannan; the resulting galactomannan–amylase complex being inactive. Processing of starch at low water levels greatly affects the catalytic efficiency of α-amylase. The K_m value for starch heat treated in limited water is raised and k_{cat} is lowered relative to starch gelatinised in excess water. Since galactomannan has no effect on the K_m of α-amylase, we conclude that the inhibitory action of the polymer is not secondary to a decrease in available water. Neither does it seem to be a consequence of impaired diffusion of enzyme, substrate and products because of an increase in viscosity of the medium. Thus, the effects of sNSP in lowering postprandial glycaemia not only involve modifications of gut physiology, but also include direct inhibition of the first stage in the biochemical degradation of starch.

Keywords: α-Amylase; Nonstarch polysaccharide; Dietary fibre; Galactomannan inhibition; Hydrothermal treatment

1. Introduction

Dietary nonstarch polysaccharides (NSP) are major constituents of dietary fibre, which is a generic term to describe indigestible substances mainly derived from the cell walls of plant foods [1]. Thus, the term dietary fibre is usually defined physiologically as the edible portions of plants that are resistant to digestion and absorption in the human small intestine [2,3]. Water-soluble NSP (sNSP) such as legume galactomannans and cereal β-glucans can result in the formation of viscous solutions in the gastrointestinal tract and delay the absorption of nutrients, e.g. glucose, in the small intestine [4–7]. The effects of sNSP on human metabolism are considered to be largely beneficial in that they decrease postprandial glycaemia and insulinaemia [8–11] and lower the fasting level of plasma cholesterol, mainly the low-density lipoprotein fraction [12–14]. One potential application of sNSP, therefore, is in the prevention and treatment of metabolic disorders. For example, guar gum, a galactomannan-rich legume flour, has received considerable attention as an oral antidiabetic agent in the treatment of mainly type 2 (noninsulin-dependent) diabetes [12,15–18].

Guar gum is derived from the seed endosperm of the Indian cluster bean (*Cyamopsis tetragonoloba* (L.) Taub) [18]. One of the important properties of guar gum is that it can be easily dispersed in water to form viscous solutions. This property is exploited commercially; for example, it is used in the food industry as a stabiliser and thickening agent in a wide variety of foods, usually at concentrations of less than 1% [19,20]. The viscosifying action of guar gum is attributed to its galactomannan content, which in commercial preparations is usually greater than 80% of the gum

* Corresponding author. Tel.: +44-20-7848-4592; fax: +44-20-7848-4802.

E-mail address: peter.butterworth@kcl.ac.uk (P.J. Butterworth).

0304-4165/02/$ - see front matter
PII: S0304-4165(02)00209-X

weight [20]. Guar galactomannan consists of an essentially linear β-D-(1 → 4) mannose backbone with irregular substituted α-D-(1 → 6)-linked galactose side groups that results in increased solubility of the polysaccharide [20,21]. The molar ratio of galactose to mannose varies slightly with the seed origin, but is approximately 0.56–0.68 [20,21].

The mechanism by which sNSP delays the absorption of glucose, and thus attenuates the postprandial rise in blood glucose, is not well understood [6,7,20]. One reason for this is that many of the preparations rich in sNSP that have been used in previous studies are poorly characterised in terms of their molecular weight and solution properties [20]. Guar galactomannan has been well characterised, however, and is therefore a useful model polysaccharide in experiments designed to elucidate the biological action of sNSP [6,20].

The blood glucose lowering effect of guar and similar sNSP observed in acute studies seems to depend mainly on the capacity of the polymer to increase the viscosity of gastric and upper intestinal contents [4–6,20–23]. The increase in viscosity can affect gastric function (e.g. emptying and sieving) and inhibit propulsive and mixing effects generated by peristalsis [20,22,24,25]. Under these conditions, therefore, where interactions between substrates and digestive enzymes are less frequent, not only is there likely to be a decrease in the rate of digestion of starch by α-amylase, but the products of amylolysis (e.g. maltose, α-limit dextrins) will almost certainly be presented to the mucosa at a slower rate [5,6,20,26]. Evidence of a direct inhibitory effect of guar galactomannan on α-amylase is lacking, however. In a limited study conducted some years ago [27], guar galactomannan was found to inhibit competitively both maltase and dipeptidase activities of intestinal everted sacs. The inhibition disappeared, however, when intestinal homogenates were used as the source of the enzymes. Given the different specificity requirements of the maltase and dipeptidase and that the various kinetic parameters were influenced by the rate of stirring of reaction mixtures, the authors of the study concluded that 'simple' competitive inhibition was unlikely and that effects on the viscosity of the medium were probably responsible for the inhibition.

In one study, we reported a decrease in the in vitro starch digestibility rate of wheat bread containing guar galactomannan compared with a control bread when incubated with porcine pancreatic α-amylase [26]. In the same study, microscopical examination of digesta removed from the jejunum of pigs fed guar-containing wheat bread revealed that the wheat starch granules were coated with a layer of galactomannan. We concluded from this that guar gum may act at the level of the food matrix as a physical barrier during starch digestion as a result of the galactomannan forming a layer around starch granules, which could then shield the latter from enzyme attack. In vivo, such 'barrier' effects would be in addition to viscosity-induced hindrance to bulk mixing, propulsion and nutrient diffusion and be expected to contribute to the blood glucose lowering action of guar.

Pancreatic α-amylase is critically involved in the initial stage of starch digestion and so we have been studying the kinetics of amylolysis [28] in seeking mechanistic explanations for the differences in the glycaemic index [18,20] of different starch-rich foods containing isoglucidic amounts of available glucose. In this paper, we report on the effects of guar galactomannan on some properties of α-amylase as part of our on-going study of seeking a molecular basis for variations in the digestibility of various native and hydrothermally treated starches.

2. Materials and methods

2.1. Reagents

Purified wheat starch with a moisture content of 9.1 ± 0.06% and normal (nonwaxy) rice starch with a moisture content of 11.2 ± 0.02% were obtained from RHM Research Ltd. (High Wycombe, Bucks, UK) and Cairn Chemical Ltd. (Chesham, Bucks. UK), respectively. Other physicochemical characteristics are listed in a previous publication [28]. A standard food grade of guar gum containing galactomannan of medium molecular weight (M90 Meyprogat) was obtained from Rhodia Food (formerly Meyhall Chemical Company AG, Kreuzlingen, Switzerland). Galactomannan was purified from the guar gum by a modification of the original method of Girhammer and Nair [29] developed in our laboratory for the extraction of a polysaccharide from a traditional plant food [30]. The guar galactomannan was extracted and purified using the method described previously [31]. This includes a maceration step after extraction of lipid with ethanol, enzyme digestion to remove starch and protein followed by ethanol precipitation of the polymer. The final step in the procedure involves freeze-drying to obtain a solid product. The purification results in a preparation that is completely free of hydrolytic enzymes and other proteins and that have improved hydration properties. The purification also improved binding of the polymer to plastic ELISA plates. The galactomannan content of the purified guar was determined using a modified procedure [31] of the method described by Englyst et al. [32]. The purity of the galactomannan was very high at 93.5% (w/w) galactomannan on a wet weight basis (moisture content of 5.6%). The galactose/mannose ratio of the polymer was 0.66 and its average molecular weight, estimated from measurements of intrinsic viscosity [30], was 1.2×10^6. Porcine pancreatic α-amylase (type 1 and claimed to be free of any contaminating proteins) and various antibodies were obtained from the Sigma Chemical Co. (Poole, Dorset, UK). The amylase preparation has approximately 1000 units of activity/mg protein (with a range, as stated by the supplier, of 700–1400 units dependent on the particular batch) where one unit is defined by the

manufacturer as releasing 1 mg maltose per 3 min from starch. The molecular weight of the enzyme is 56000. General laboratory chemicals were purchased from BDH Laboratory Supplies (Poole, Dorset, UK) and were the best grades available.

2.2. Determination of the amylose content of starches

For amylose estimation, 0.1 ml of a 1% suspension of starch was solubilised in 95% dimethylsulphoxide by heating for 15 min at 100 °C and then added to 5 ml of 0.5% (v/v) trichloracetic acid and mixed. A 50 μl volume of 0.01 N I_2–KI solution was added followed by immediate mixing. The colour was allowed to develop over 30 min and the absorbance at 620 nm was then determined [28]. The amylose concentration was calculated by multiplying the absorbance values, suitably corrected for dilution factors, by 45.8 as recommended by Chrastil [33]. The percentage amylose content of the starches was 27.5 and 16.1 for wheat and rice, respectively.

2.2.1. Preparation of polysaccharide suspensions

Wheat and rice starches were suspended in PBS at pH 7.4 and treated exactly as described by Slaughter et al. [28]. Such suspensions represent conditions in which water is in excess. To simulate the low moisture conditions used in the preparation of many starchy food products, distilled water was added accurately and with continuous gentle stirring, to native wheat starch in the proportion of 0.4 ml/g starch. The suspensions were heated in open (to allow evaporation) or sealed (to prevent evaporation) containers in an air oven at 100 °C for 20 min with constant mixing. The samples were then cooled for 5 min in an incubator at 37 °C before being diluted in PBS to provide the range of substrate concentrations required for the experiments. The moisture contents of the samples were determined immediately by a routine oven-drying method (16 h at 103 °C) for duplicate samples [34], before dilution in PBS. The water content was 10% and 40% (w/w), respectively, for samples that had been open or sealed during the initial oven heating process at 100 °C. In every case, suspensions were prepared fresh for each experiment and ranged in concentration from 0.05% to 1.0% according to experiment.

Guar galactomannan solutions were prepared in PBS at double the required final concentration (0.075–1.2% according to experiment) and allowed to hydrate for at least 18 h at room temperature (20 °C). This extended hydration procedure was found to decrease the proportion of insoluble material compared with shorter hydration periods used by other workers [31,32].

For wheat starch–galactomannan mixtures, suspensions of starch and galactomannan solutions were mixed in equal quantities and transferred to 100 ml conical flasks. The flasks were sealed with a glass marble to reduce loss by evaporation and agitated gently by swirling for 20 min either at room temperature or in a water bath at 100 °C (± 1 °C).

2.3. Assay of starch hydrolysis catalysed by porcine pancreatic amylase

Aliquots (3 ml) of the wheat starch or wheat starch–galactomannan mixtures were transferred to 6 ml Falcon tubes placed in a rotating table to provide end-over-end mixing in an incubator at 37 °C. After 30 min, 10 μl of an α-amylase solution in PBS containing 0.1 mg/ml BSA was added to the tubes. At 30-s intervals up to 10.5 min, aliquots of 0.2 ml were removed from each reaction tube and immediately microfuged to sediment undigested starch and other polysaccharides. The supernatant was then transferred to a clean microfuge tube and placed in boiling water to inactivate the enzyme. The samples were then frozen for later analysis of reducing sugar. The reaction mixtures usually contained 0.033 units/ml (0.5–0.9 nM enzyme), but for some experiments in which the effect of galactomannan was being studied, the amount of enzyme in the assay reaction mixture was increased to 0.33 units/ml (5–9 nM). The quantity of enzyme used in each case is given in the legends to the respective tables and figures. Preliminary experiments established that prior mixing of the enzyme with galactomannan for timed periods did not affect the degree of inhibition. One unit, as defined by the suppliers, will liberate 1 mg of maltose from starch in 3 min. This unit does not accord with recommended practice and so catalytic activities in our experiments were expressed as micromolar of maltose formed per minute. Our units can be converted to nanokatal by multiplying by a factor of 0.05 [28].

2.4. Determination of reducing sugar

Reducing sugar released during amylolysis was determined using the extremely sensitive Prussian Blue assay used in our previous studies [28] by reference to a standard curve obtained with maltose up to a concentration of 10 μM. Maltose was used as the reference sugar because it is a main source of reducing equivalents in the reaction products. Also, it has been customary for very many years to express amylase activity in terms of the rate of release of maltose both in the scientific literature and by commercial suppliers of the enzyme.

When galactomannan was present in reaction mixtures, the method proved unsuitable, however, because the polysaccharide seems to become partially hydrolysed during the procedure required for colour development and the released reducing sugars result in high blank readings. In preliminary experiments, attempts were made to remove galactomannan prior to colour development by precipitation with ethanol but this proved unreliable. Thus, reducing sugar was estimated in guar-containing mixtures and in nonguar controls by the 3,5,-dinitrosalicylic acid (DNS) method. Because this method is much less sensitive than the Prussian Blue one, the enzyme concentration in the assays for certain experiments had to be increased accordingly (see above), but high blank values were avoided. The 200 μl sample taken from

the reaction mixture was diluted to 250 μl with PBS and combined with 125 μl of 3 M NaOH, 125 μl water and 500 μl of DNS solution [35] and vortexed. The tubes were then placed in boiling water for 10 min exactly and then allowed to cool to room temperature before the addition of 5 ml of water. The absorbance was measured at 530 nm after standing for 1 h at room temperature. Mixtures containing known concentrations of maltose up to 3 mM were used to prepare standard curves from which the concentration of maltose produced in the enzyme reactions could be calculated.

2.5. Microscopy

Samples of wheat starch and wheat starch–galactomannan mixtures were examined by normal and polarised light microscopy. For electron microscopy, native polysaccharide samples were mounted on aluminium microscopy stubs using sticky tabs. For samples that had received the heat treatments described above, approximately 150 μl was pipetted onto the stub and after settlement of the solid material, excess fluid was removed from the surface. The stub was then plunged into dichlorodifluoromethane (Freon) cooled in liquid nitrogen [36] and placed in a freeze drier overnight to freeze the starch directly onto the aluminium stub. The stubs were then sputter coated with gold before being examined and photographed using a Philips 501 B Scanning Electron Microscope (SEM).

2.6. Binding studies

Water was preheated to approximately 45 °C and purified guar galactomannan was gently added, with continuous stirring, in small quantities to provide a concentration range up to and including 1% by weight in the final solution (i.e., corresponding to 0.94% galactomannan when allowing for the moisture content of the starting material). The mixtures were heated to 80 °C approximately for 5 min and then allowed to cool to room temperature with stirring maintained throughout. Sufficient water was then added to bring the concentration of galactomannan to that required for the experiments and to allow complete hydration.

To the wells of a Dynatech Immulon (polycarbonate) or a Falcon (PVC) microtitre plate was added 200 μl of the galactomannan solutions to produce a range of amounts of polymer across the plate. Control wells contained 200 μl of water. The different plastics used in their manufacture affect the hydrophilicity of the plates and it was hoped that adherence of the polysaccharide would be favoured by one or other of the plastics. The plate was dried by placing it for 6 h in a fan-assisted oven at 70 °C. The plate was then blocked by overnight treatment at room temperature with 200 μl 1% BSA (w/v) dissolved in PBS containing 0.05% (v/v) Tween 20 (PBST) added to each well. The plate was kept in a damp box. The wells were emptied and washed three times with the PBST solution and then kept at 37 °C for approximately 40 min to allow the wells to dry. α-Amylase was diluted in PBST to give a concentration of approximately 0.03 mg protein/ml and then a 200 μl aliquot was added to each well, i.e., approximately 6.6 units of activity per well. The plate was allowed to stand in a damp box for 3 h at 4 °C and then washed once with PBST followed by drying. Any α-amylase bound within the wells was detected by a conventional ELISA technique using rabbit anti-human α-amylase (which is known from the manufacturer's report to cross-react with porcine enzyme) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. Alkaline phosphatase activity was detected by addition of a 1 mg/ml solution of *p*-nitrophenyl phosphate dissolved in 0.1 M diethanolamine–HCl buffer pH 9.0. For control purposes, i.e., to detect nonspecific binding of the antibodies, wells were set up that contained all the reactants except for either α-amylase or the primary antibody. No reaction was detected in the absence of amylase or the primary antibody.

Post-assay, the plates were stained with 0.1% (w/v) Toluidine Blue, which is a nonspecific stain used for identifying polysaccharide gums [26], to test for the presence of galactomannan bound into the wells.

2.7. Analysis of kinetic data

Weighted nonlinear regression fits to the Michaelis–Menten equation [37] of reaction rates measured at different substrate concentrations was used to determine the kinetic parameters. A similar treatment was applied to the inhibition data.

3. Results

Light microscopic examination under polarized light (results not shown) of hydrothermally treated (100 °C) mixtures revealed that when the galactomannan accounted for one-third of the total solids in the original preheated mixture (i.e., 1 g starch plus 0.5 g galactomannan per 100 ml), gelatinisation of the starch was incomplete. This conclusion was based on the finding that there was retention of birefringence by some of the starch granules. Also, material leached from granules during treatment appeared to be distributed fairly evenly throughout the sample. Based on previous knowledge and experimental findings, the bulk of the leached material is assumed to be amylose [28,38]. Granule remnants rich in amylopectin were also visible. When viewed by polarised light, birefringent 'B' granules could be seen; an indication that some of the starch granules had not gelatinised, perhaps because the hydrophilic galactomannan had restricted the availability of water for starch swelling. SEM pictures of native starch and of granules heated at 100 °C under limited and excess moisture conditions are shown in Fig. 1. Granules heated in excess moisture were fully gelatinised, as expected. When the

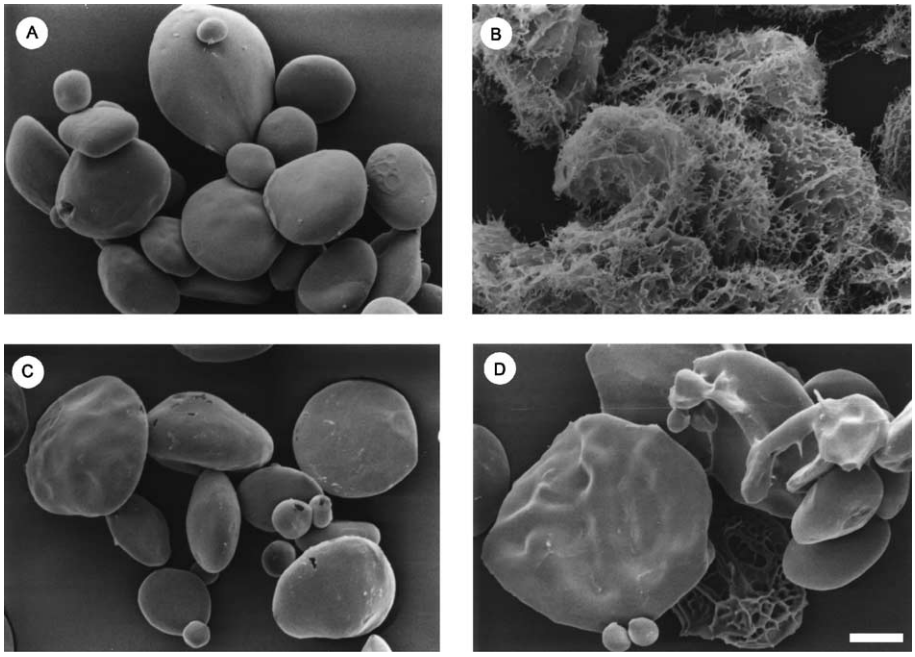

Fig. 1. SEM micrographs of native wheat starch granules and of samples heated at 100 °C. (A) Native starch granules; (B) after heating in excess moisture (i.e., fully gelatinised); (C) after heating in an open container, which resulted in a final moisture content of 10% (w/w); (D) after heating in a closed container, which resulted in a final moisture content of 40% (w/w). All of the figures are of identical magnification and the bar in D represents 10.6 μm.

moisture content was 10%, the starch granules appeared to be largely intact although slightly distorted perhaps. At 40% moisture, heating resulted in some swollen and deformed granules that seemed to lack the disrupted appearance characteristic of fully gelatinised starch shown in Fig. 1B. Some fully gelatinised material was also present, however.

When the enzyme was acting on 1% (w/v) native starch, a significant effect of galactomannan on the hydrolysis rate could be demonstrated at concentrations of polymer in excess of 1%. These reaction mixtures were viscous and difficult to pipette accurately. In addition, mixing of the reactants during the assays by continuous rotation was not very effective and so detailed kinetic experiments under conditions of high viscosity were not attempted. In kinetic experiments where the effect of 0.5% galactomannan was tested on amylase acting on a range of concentrations of gelatinised wheat starch, an inhibitory action was demonstrated. K_m was not affected significantly by the presence of guar, but k_{cat}/K_m decreased by 33% (Table 1). Such action would normally be interpreted as a noncompetitive effect of guar galactomannan on starch hydrolysis. Fig. 2 shows a Hanes (S vs. S/v) plot of data obtained with wheat starch thermally treated in the presence of galactomannan and it can be seen that the pattern of inhibition is classically noncompetitive. Treating the data as such allows calculation of a K_i value for galactomannan of approximately 0.5% when it is present in heat-treated mixtures of wheat starch and galactomannan in excess water.

Fig. 3 shows Dixon ($1/v$ vs. I) plots of some data obtained for the inhibition by galactomannan of amylase acting on native and gelatinised nonwaxy rice starch. This kind of plot is useful for detection of multiple modes of inhibition; deviation from linearity suggests complex behaviour. We chose to examine the inhibition phenomena using a starch that differed from wheat in physical characteristics and amylose content [28]. By so doing, we thought that we

Table 1
Kinetic parameters for porcine pancreatic α-amylase acting on wheat starch substrates

Substrate	V_{max} (μM min^{-1})	K_m (%)	k_{cat} (min^{-1} × 10^5)	k_{cat}/K_m (× 10^5)
Native starch	11.03 ± 1.11	0.51 ± 0.08	0.14 ± 0.016	0.27 ± 0.029
Gelatinised starch	136.7 ± 3.6	0.13 ± 0.00	1.66 ± 0.051	12.51 ± 0.30
Low moisture (10%)	12.97 ± 0.78	1.29 ± 0.09	0.16 ± 0.01	0.12 ± 0.00
Low moisture (40%)	72.4 ± 3.02	1.92 ± 0.10	0.89 ± 0.04	0.46 ± 0.00
Heat-treated starch/galactomannan[a]	61.63 ± 1.9	0.09 ± 0.01	0.76 ± 0.02	8.44 ± 0.65

The low moisture samples were obtained by heating at 100 °C (in the presence of 0.4 ml water/g starch) in open or sealed containers, which resulted in final moisture contents of 10% and 40%, respectively. Fully gelatinised samples were obtained by heating starch at 100 °C for 20 min in excess water. The galactomannan–starch mixture was treated in the same way as the fully gelatinised. The parameters were determined from assay mixtures containing 0.033 units/ml amylase.

[a] The DNS method for reducing sugar was used here.

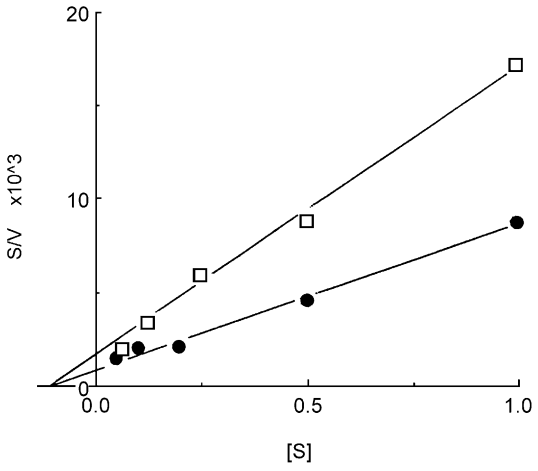

Fig. 2. Hanes (S/v against S) plot of galactomannan inhibition of α-amylase action on gelatinised wheat starch. Galactomannan was present at concentrations of 0 (●); and 0.5% (□). Gelatinised starch and gelatinised starch–galactomannan mixtures were added to reaction tubes to provide a range of starch concentrations in the presence of galactomannan at a fixed concentration of 0.5%. The assays were initiated by the addition of 0.033 units/ml enzyme and initial rates were determined from samples taken at 2-min intervals. The plots are visual fits but the K_m values calculated by nonlinear regression fitting to the Michaelis–Menten equation were $0.134 \pm 0.042\%$ and $0.083 \pm 0.18\%$ for starch alone and (starch + guar), respectively. The equivalent values for V_{max} were 135.6 ± 13.3 and 65 ± 3.8 μM min^{-1}.

could gain some information about whether the inhibition of amylase activity is attributable mainly to starch–galactomannan interaction [26] rather than an effect on the enzyme itself. In contrast to the experiments with wheat starch, described above, the galactomannan was not included in suspensions of rice starch undergoing gelatinisation but was added to the assay mixtures just before initiation of the reaction by the addition of amylase. The Dixon plots are essentially linear and therefore suggestive of a single mode of interaction with the enzyme. The K_i values, calculated by a weighted fit of the data, were $0.37 \pm 0.1\%$ and $0.53 \pm 0.2\%$ for native and gelatinised, respectively, i.e., not significantly different from each other and essentially identical to the value obtained for galactomannan acting on wheat starch. The plots intersect on the negative x-axis and are indicative of noncompetitive inhibition irrespective of whether native or gelatinised starch is used as the substrate. No evidence was obtained in preliminary experiments to suggest that the onset of inhibition is time dependent and so any galactomannan–amylase interaction must occur relatively rapidly.

The water content of starch suspensions greatly affects the kinetic properties of α-amylase. As shown in Table 1, at low moisture contents, the K_m for starch hydrolysis is increased by a factor of 2.5- to 4-fold relative to native starch suspended in excess water. For starch with 10% moisture, V_{max} is low relative to fully gelatinised wheat starch, but when the moisture content was 40%, V_{max} was 53% of the fully gelatinised value. A comparison with gelatinised starch is made because the heating protocol during the production of the starch suspensions of low moisture content was comparable with the method used for gelatinising starch in excess water. Catalytic efficiency as expressed by k_{cat}/K_m ratios indicates that starch heated in conditions of limited water is a poorer substrate than fully gelatinised starch, but does not differ widely from native starch. Thus, a rise in K_m is partially compensated by a rise in V_{max}. It is noteworthy that the k_{cat}/K_m value for starch gelatinised in excess moisture, but in the presence of galactomannan, is much greater than the values for starch in limited water (Table 1).

Fig. 4 shows the results of a binding experiment using two types of ELISA plate. As the quantity of galactomannan bound to the wells increases, the absorbance at 405 nm originating from the release of *p*-nitrophenol catalysed by the alkaline phosphatase–anti-IgG conjugate also increases. The increase is not linear with the polymer content of the wells, but appears to show a threshold phenomenon such that binding of amylase increases very sharply between 0.3 and 0.6 mg of galactomannan to reach a plateau between 0.6 and 1 mg. It must be stressed, however, that the polymer contents described in the figure are the amounts *originally* added into the wells, but because of the relatively low adhesion of galactomannan to the plastic, much of the polymer seems to be lost during the washing stages of the ELISA. A lack of firm adhesion of galactomannan was evident with both types of plates tested. Therefore, unless at least 0.5 mg is added to the wells initially, an insufficient amount remains after the washings to permit detection of galactomannan–amylase interaction. Staining with Toluidine Blue subsequent to the ELISA assay confirmed that a strong staining for galactomannan occurred only in those wells that had registered a strong alkaline phosphatase

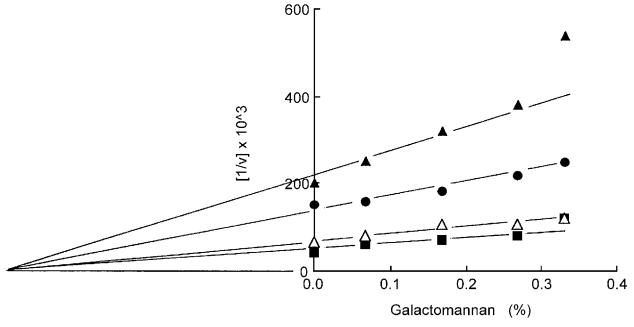

Fig. 3. Dixon ($1/v$ against I) plot for galactomannan inhibition α-amylase action on native and gelatinised normal rice starch. Native starch at 0.3% concentration (■); native starch at 0.15% concentration (▲); gelatinised starch at 0.3% concentration (△); gelatinised starch at 0.15% concentration (●). Galactomannan was added to the reaction mixtures to achieve the required concentrations. The reaction mixtures contained 0.33 units of enzyme activity. All values on the y-axis for the gelatinised samples have been multiplied by a factor of 10 for ease of plotting all the data within one set of axes. The lines through the data points are a visual fit to a common intercept on the x-axis at -0.4, but K_i values for galactomannan quoted in the text of the paper were obtained by weighted regression using weights of v^4 [37].

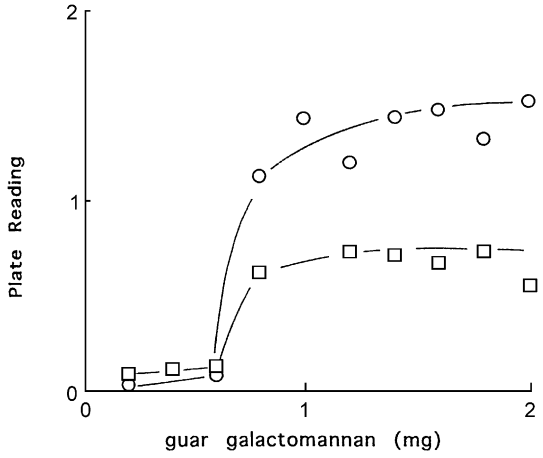

Fig. 4. Direct binding of α-amylase to galactomannan. The figure shows the results of single, but typical experiments using the Dynatech (polycarbonate) plate (○) and a Falcon (PVC) plate (□). Enzyme that became bound to the polymer was detected by using an antibody directed against human pancreatic α-amylase followed by alkaline phosphatase-conjugated anti-rabbit IgG. The phosphatase activity was monitored using *p*-nitrophenyl phosphate as substrate.

signal. Thus, although it can be concluded that α-amylase binds to galactomannan, reliable calculations of binding affinities and/or estimates of the binding density, i.e., the moles of amylase bound per mole of galactomannan, cannot be made from our data.

4. Discussion

In our experiments, among the factors that can be considered to have an influence on the catalytic efficiency of α-amylase are (a) the physical access of the starch to the enzyme, (b) the availability of water needed for the hydrolysis of the glycosidic linkages in starch, (c) lowered rates of diffusion of substrate, enzyme and products because of a relatively high viscosity of the reaction medium (galactomannan and starch) and (d) nonproductive binding of amylase to galactomannan. Each of these factors is not necessarily unrelated to one or more of the others.

The kinetic data reveal that K_m values are not affected significantly by the presence of galactomannan and suggest that starch–amylase interaction is not compromised by the presence of this polymer. Studies in vivo have demonstrated that guar gum can become attached to the surface of starch granules [26], but the lack of effect on K_m and a lack of time dependency of inhibition implies that there are still sufficient unmasked attack sites on the granule where amylase can act. The existence of a significant physical barrier to the enzyme resulting from the galactomannan–starch interaction therefore appears unlikely under the conditions selected for our experiments.

Galactomannan acts as a noncompetitive inhibitor of α-amylase, i.e., K_m is unaffected by the presence of the polymer but k_{cat} and hence k_{cat}/K_m are decreased. A fall in k_{cat} could result from decreased water availability and/or lowered rates of diffusion of products. The diffusion of reactants and hydrolysis products may be influenced by a change in the rheology of the incubation mixture since galactomannan increases the viscosity of water markedly at relatively low polymer concentrations (0.5–1.0%, w/w). In fact, a reaction mixture containing both galactomannan and starch will be much more viscous than a solution containing starch only [31]. Rheological measurements of the critical overlap concentration (Ccr), which is the point at which the viscosity increases dramatically with increase in concentration of the solute [39], show that this occurs at 0.24% approximately in solutions of galactomannan (molecular weight of 1.3 million). The corresponding concentration (Ccr) for galactomannan–starch mixtures is expected to be lower than this, i.e., at the lower end of the range of mixtures used in our experiments. Because the K_m value is unaffected by galactomannan, it can be argued that viscosity effects are of little significance in our studies. To place this argument in enzyme kinetic terms, it is worth remembering that the 'on constant' (k_{on}) for substrate–enzyme binding appears as a term in the denominator of expressions for K_m. The rate constant (k_{on}) is very dependent on the collision rate of the enzyme and substrate and is expected to decrease with a rise in viscosity of the medium. A fall in k_{on} would be likely to produce a rise in K_m; something that is not seen in our guar experiments. Thus, diffusion per se does not seem to account for the actions of galactomannan on amylase action. As part of a previous study of factors influencing gastric lipase activity, it was shown that increased viscosity induced by guar gum had no affect on catalytic activity unless the assay was continued for sufficient time to allow the build-up of product [40]. It has also been shown that diffusion of Na^+ ions is not affected by increased viscosity generated by guar gum [23]. Therefore, it seems reasonable to conclude that the diffusion of the product maltose, a relatively small molecule and a well recognised inhibitor of amylase, would be little affected by the presence of galactomannan at the concentrations used in our experiments.

The raised K_m for starch heated in low moisture conditions seems to point to the importance of water availability. Although starch granules become swollen, they are rendered less favourable for amylolysis, which suggests adoption of a more ordered and thus resistant structure. With a water content of 40%, a rise in k_{cat} begins to compensate for the unfavourable K_m and therefore catalytic efficiency is improved relative to native starch. Nevertheless, the catalytic efficiency remains low compared with fully gelatinised starch. That the K_m values for both native and gelatinised starch are unaffected by the presence of galactomannan can be interpreted as evidence that the action of this polymer cannot be fully explained by assuming that it limits the availability of water.

Noncompetitive inhibition could result from absorption of α-amylase to galactomannan. A region on the enzyme [28,41,42] could possess some affinity for NSP. Direct

binding of amylase to galactomannan has been demonstrated in our experiments although the affinity could not be determined. Previous studies of inhibition of α-amylase by polysaccharides have demonstrated that the system is not simple. For example, cyclodextrins can inhibit in a competitive mode or a noncompetitive one according to whether the substrate is amylose or maltopentaose [43], and the authors of this report suggest that cyclodextrin binds at a secondary site that is accessible only after maltopentaose becomes bound to the active site. Whether the putative site for cyclodextrin is relevant to our findings with galactomannan is unknown and calls for further study.

The K_i value of galactomannan (0.32–0.5%) calculated assuming classical noncompetitive inhibition, and which therefore represents a dissociation constant, is almost identical with the K_m value for amylase acting on native wheat starch [28]. With an average molecular weight of 1.2×10^6, however, a 0.4% solution of galactomannan represents a concentration of approximately 3.3 μM, which is about two orders of magnitude greater than the K_m for the enzyme acting on potato amylose [43]. Thus, although galactomannan seems relatively potent as an inhibitor, its affinity for α-amylase is low compared with a starch substrate.

We conclude that the effects of guar gum in lowering postprandial glycaemia and insulinaemia not only involve actions on gut physiology as outlined in the Introduction, but also include a direct inhibition of the first stage in the biochemical degradation of starch, i.e., amylolysis

Acknowledgements

The authors acknowledge the financial support of the Ministry of Agriculture, Fisheries and Food (studentship for S.L.S.) and SmithKline Beecham. We thank Professor S. Ross-Murphy for helpful discussions during the preparation of this paper and Dr. A. Brain for expert assistance with the electron microscopy. Thanks are also due to Ms. Asako Shida for technical help with some of the kinetic experiments.

References

[1] C.T. Brett, K.W. Waldron, Physiology and Biochemistry of Plant Cell Walls, 2nd edn., Chapman & Hall, London, 1996.
[2] H.C. Trowell, D.A.T. Southgate, T.M.S. Wolever, A.R. Leeds, M.A. Gassull, D.J.A. Jenkins, Dietary fibre redefined, Lancet i (1976) 967.
[3] D.A.T. Southgate, Current versus new definitions of fibre, in: M. Hill (Ed.), The Right Fibre for the Right Disease, The Royal Society of Medicine Press, London, 1999, pp. 33–42.
[4] C. Cherbut, E. Albina, M. Champ, J.L. Doublier, G. Lecannu, Action of guar gum on the viscosity of digestive contents and on gastrointestinal motor function in pigs, Digestion 4 (1990) 205–213.
[5] P.R. Ellis, F.G. Roberts, A.G. Low, L.M. Morgan, The effect of high-molecular weight guar gum on net apparent glucose absorption and net apparent insulin and gastric inhibitory polypeptide production in the growing pig: relationship to rheological changes in jejunal digesta, Br. J. Nutr. 74 (1995) 539–556.
[6] P.R. Ellis, P. Rayment, Q. Wang, A physico-chemical perspective of plant polysaccharides in relation to glucose absorption, insulin secretion and the entero-insular axis, Proc. Nutr. Soc. 55 (1996) 881–898.
[7] P.J. Wood, Cereal β-glucans: structure, properties and health claims, in: B.V. McCleary, L. Prosky (Eds.), Advanced Dietary Fibre Technology, Blackwell, Oxford, UK, 2001, pp. 315–327.
[8] D.J.A. Jenkins, A.R. Leeds, M.A. Gassull, T.M.S. Wolever, D.V. Goff, K.G.M.M. Albertini, T.D.R. Hockday, Unabsorbable carbohydrates and diabetes: decreased post-prandial hyperglycaemia, Lancet ii (1976) 172–174.
[9] P.R. Ellis, A.C. Apling, A.R. Leeds, N.R. Bolster, Guar bread: acceptability and efficacy combined. Studies on blood glucose, serum insulin and satiety in normal subjects, Br. J. Nutr. 46 (1981) 267–276.
[10] L.M. Morgan, J.A. Tredger, J. Wright, V. Marks, The effect of soluble- and insoluble-fibre supplementation on post-prandial glucose tolerance, insulin and gastric inhibitory polypeptide secretion in healthy subjects, Br. J. Nutr. 64 (1990) 103–110.
[11] S.J. Gatenby, P.R. Ellis, L.M. Morgan, P.A. Judd, Effect of depolymerised guar gum on acute metabolic variables in patients with non-insulin dependent diabetes, Diabet. Med. 13 (1996) 358–364.
[12] D.B. Peterson, P.R. Ellis, J.M. Baylis, P. Fielden, J. Adjodhia, A.R. Leeds, E.M. Jepson, Low dose guar in a novel food product: improved metabolic control in non-insulin dependent diabetes, Diabet. Med. 4 (1987) 111–115.
[13] J. Tuomiliehto, M. Silvastri, A. Aro, A. Koistinen, P. Karttunen, C.-G. Gref, C. Ehnholm, M. Uusitupa, Long term treatment of severe hypercholesterolaemia with guar gum, Atherosclerosis 72 (1988) 157–162.
[14] D.E. Blake, C.J. Hamblett, P.J. Frost, P.A. Judd, P.R. Ellis, Wheat bread supplemented with depolymerised guar gum reduces plasma cholesterol concentration in hypercholesterolaemic human subjects, Am. J. Clin. Nutr. 65 (1997) 107–113.
[15] A. Aro, M. Uusitupa, E. Voutilainen, K. Hersio, T. Korhonen, O. Siitonen, Improved diabetic control and hypocholesterolaemic effect induced by long term dietary supplementation with guar gum in type 2 (insulin-independent) diabetes, Diabetologia 21 (1981) 29–33.
[16] H.S. Fussell, G. Williams, T.E. Adrien, S.R. Bloom, Guar sprinkled on food: effect on glycaemic control, plasma lipids and gut hormones in non-insulin dependent diabetic patients, Diabet. Med. 4 (1987) 463–468.
[17] M. Rendell, Dietary treatment of diabetes mellitus (editorial), N. Engl. J. Med. 342 (2000) 1440–1441.
[18] P.R. Ellis, The effect of fibre on diabetes, in: M. Hill (Ed.), The Right Fibre for the Right Disease, The Royal Society of Medicine Press, London, 1999, pp. 33–42.
[19] R.L. Whistler, T. Hymowitz, Guar: Agronomy, Production, Industrial Use and Nutrition, Purdue University Press, West Lafayette, IN, 1979.
[20] P.R. Ellis, Q. Wang, P. Rayment, Y. Ren, S.B. Ross-Murphy, Guar gum: agricultural and botanical aspects, physico-chemical and nutritional properties and its use in the development of functional foods, in: S.S. Cho, M. Dreher (Eds.), Handbook of Dietary Fibre, Marcel Dekker, New York, 2001, pp. 613–657.
[21] I.C.M. Dea, A. Morrison, Chemistry and interactions of seed galactomannans, Adv. Carbohydr. Chem. Biochem. 31 (1975) 241–312.
[22] N.A. Blackburn, J.S. Redfern, H. Jarjis, A.M. Holgate, I. Hemming, J.H.B. Scarpello, I.T. Johnson, N.W. Read, The mechanism of action of guar gum in improving glucose tolerance in man, Clin. Sci. 66 (1984) 329–333.
[23] C.A. Edwards, I.T. Johnson, N.W. Read, Do viscous polysaccharides slow absorption by inhibiting diffusion or convection, Eur. J. Clin. Nutr. 42 (1998) 307–312.
[24] L. Bueno, F. Praddaude, J. Fioramonte, Y. Ruckesbusch, Effect of dietary fibre on gastrointestinal motility and jejunal transit time in dogs, Gastroenterology 80 (1981) 701–707.
[25] N.W. Read, M.A. Eastwood, Gastro-intestinal physiology and function, in: T.F. Schweizer, C.A. Edwards (Eds.), Dietary Fibre—A Component of Food, Springer-Verlag, London, 1992, pp. 103–117.

[26] C.S. Brennan, D.E. Blake, P.R. Ellis, J.D. Schofield, Effect of galactomannan on wheat bread microstructure and on the in vitro and in vivo digestibility of starch in bread, J. Cereal Sci. 24 (1996) 151–160.
[27] B. Elsenhans, U. Sufke, R. Blume, W.F. Caspary, In vitro inhibition of rat intestinal surface hydrolysis of disaccharidases and dipeptidases by guaran, Digestion 21 (1981) 98–103.
[28] S.L. Slaughter, P.R. Ellis, P.J. Butterworth, An investigation of the action of porcine pancreatic α-amylase on native and gelatinised starches, Biochim. Biophys. Acta 1525 (2001) 29–36.
[29] U. Girhammer, B.M. Nair, Isolation, separation and characterisation of water soluble non-starch polysaccharides from wheat and rye, Food Hydrocoll. 6 (1992) 285–299.
[30] Q. Wang, P.R. Ellis, S.B. Ross-Murphy, J.S.G. Reid, A new polysaccharide from a traditional Nigerian plant food: *Detarium senegalense* Gmelin, Carbohydr. Res. 284 (1996) 229–239.
[31] P. Rayment, S.B. Ross-Murphy, P.R. Ellis, Rheological properties of guar galactomannan and rice starch mixtures—I. Steady shear measurements, Carbohydr. Polym. 28 (1996) 121–130.
[32] H. Englyst, M.E. Quigley, G.J. Hudson, J.H. Cummings, Determination of dietary fibre as non-starch polysaccharides by gas–liquid chromatography, Analyst 117 (1992) 1707–1714.
[33] J. Chrastil, Improved colorimetric determination of amylose in starches or flours, Carbohydr. Res. 159 (1987) 154–158.
[34] R.S. Kirk, R. Sawyer, Pearson's Composition and Analysis of Foods, 9th edn., Churchill Livingstone, Edinburgh, UK, 1991.
[35] D.T. Plummer, An Introduction to Practical Biochemistry, 3rd edn., McGaw-Hill, Maidenhead, UK, 1987, pp. 180–181.
[36] P. Bowler, A.D. Evers, J. Sarjent, Dehydration artifacts in gelatinised starches, Starch/Starke 39 (1987) 46–49.
[37] G.N. Wilkinson, Statistical estimations in enzyme kinetics, Biochem. J. 80 (1961) 324–332.
[38] R.F. Tester, Starch: the polysaccharide fractions, in: P.J. Frazier, P. Richmond, A.M. Donald (Eds.), Starch Structure and Functionality, The Royal Society of Chemistry, Cambridge, UK, 1997, pp. 163–171.
[39] S.B. Ross-Murphy, Rheological methods, in: S.B. Ross-Murphy (Ed.), Physical Techniques for the Study of Food Biopolymers, Blackie Academic and Professional, London, UK, 1994, pp. 343–392.
[40] B. Pasquier, M. Armand, C. Castelain, F. Guillon, P.H. Borel, H. Lafont, D. Lairon, Emulsification and lipolysis of triacylglycerols are altered by viscous soluble dietary fibres in acidic gastric medium in vitro, Biochem. J. 314 (1996) 269–275.
[41] G.D. Brayer, L. Yaoguang, S.G. Withers, The structure of human pancreatic α-amylase at 1.8 Å resolution and comparisons with related enzymes, Protein Sci. 4 (1995) 1730–1742.
[42] S.B. Larson, A. Greenwood, D. Casio, J. Day, A. McPherson, Refined molecular structure of pig pancreatic alpha-amylase at 2.1 Å resolution, J. Mol. Biol. 235 (1994) 1560–1584.
[43] R. Koukiekolo, V. Desseaux, Y. Moreau, G. Marchis-Mouren, M. Santimone, Mechanism of porcine pancreatic α-amylase: inhibition of amylose and maltopentaose hydrolysis by α-, β-, and γ-cyclodextrins, Eur. J. Biochem. 268 (2001) 841–848.