Carbohydrate Polymers **12** (1990) 155–168

Development of a Biotechnological Process for the Modification of Galactomannan Polymers with Plant α-Galactosidase

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(Received 10 November 1988; revised version received and accepted 6 February 1989)

ABSTRACT

Two galactomannan polysaccharides (guar gum, locust bean gum) are commonly used in foods and other applications to manipulate aqueous rheology. Locust bean gum (23% galactose, 77% mannose) is more functional and more expensive than guar gum (38% galactose, 62% mannose). We have investigated the enzymic (α-galactosidase) removal of side-chain galactose residues from guar gum to yield galactomannans similar in chemical composition and functional properties to locust bean gum. The optimum concentration for α-galactosidase action increased from ~ 2% w/w (solution state) at 35°C to ~ 10% w/w (gel-like state) at 42°C to 20–30% w/w (semi-solid particulate state) at 50°C due to increased enzymic temperature tolerance at high substrate concentrations. Galactomannans varying in galactose content were prepared by manipulating reaction time, temperature and enzyme/guar gum ratio. Enzymically modified guar galactomannans with 22–24% galactose contents were found to reproduce the rheological and stabilisation properties of locust bean gum. These findings form the basis for a feasible biotechnological route for the upgrading of guar gum to galactomannan polymers with enhanced functionality.

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INTRODUCTION

Polysaccharides are a major constituent of vegetable matter and are an important renewable raw material resource. Biotechnological processes have been used to exploit these abundant raw materials, well known examples being the enzymic or microbial degradation of cellulose and starch to produce e.g. ethanol and glucose. In addition to such depolymerisation reactions, there is considerable scope for non-depolymerising enzymic modifications as a means of producing polymeric materials with increased value. Several useful enzyme-mediated modifications of polysaccharides have been demonstrated in the laboratory (McCleary, 1986; Skjak-Braek *et al.*, 1986) but we are not aware of any commercial operations based on these processes. One example of an enzymic polysaccharide modification is the action of α-galactosidase on galactomannans (McCleary, 1986). The development of conditions appropriate to large-scale operation of this process is the subject of the present report.

Galactomannans are the energy reserve polysaccharide in seeds of all endospermic leguminous plants (Meier and Reid, 1983); they consist of a polymeric chain of $(1\rightarrow4)$-β-linked D-mannosyl residues substituted by single D-galactosyl residues α-linked to mannosyl C-6 sites. The ratio of galactose to mannose residues depends on the botanical source and can range from *c.* 50:50 (i.e. full substitution) to *c.* 15:85 (Dea and Morrison, 1975). Two galactomannans are commercially produced in large quantities, guar gum (38% galactose, 62% mannose) from guar (*Cyamopsis tetragonolobus*) seeds and locust bean gum (23% galactose, 77% mannose) from carob (*Ceratonia siliqua*) seeds. Guar is an annual plant which grows in semi-arid conditions and is indigenous to the Indian sub-continent. Carob trees fruit (i.e. produce galactomannan) only after several years' growth and take more than 10 years to reach maturity. They are grown in Mediterranean climates but, owing to the long maturation period, labour-intensive harvesting, and competition from tourism and other cash crops, ageing carob plantations may not be replaced in the future.

The useful commercial properties of galactomannans are derived from two features of their molecular structure: (1) the β-$(1\rightarrow4)$ mannan backbone is relatively rigid and leads to high viscosities in dilute aqueous solution (Robinson *et al.*, 1982); (2) for galactomannans of low ($<30\%$) galactose content, elastic gels are formed in combination with other polysaccharides (e.g. carrageenan, agar, xanthan), and frozen multi-phase food systems such as ice cream can be effectively stabilised (Dea and Morrison, 1975). For galactomannan applications involving aqueous

viscosity enhancement, either guar gum or locust bean gum could be used, but guar gum is the material of choice for economic reasons. However, for applications involving e.g. co-gelation with other polysaccharides or ice cream stabilisation, locust bean gum is used since guar gum does not exhibit the desired properties. Owing to the relatively high cost of locust bean gum and the possibility of diminishing supplies in the future, it is of commercial interest to determine whether the cheaper and more readily produced guar gum can be converted into a material having the desirable functional properties of locust bean gum.

To convert guar galactomannan into a locust bean galactomannan equivalent, some of the side-chain (1→6)-α-linked D-galactosyl residues need to be removed without significant cleavage of the backbone. The required specificity is only likely to be achieved in an enzyme-mediated reaction. The appropriate enzyme, α-galactosidase, is one of the group of enzymes utilised in mobilising galactomannan energy reserves during seed germination; it has been extracted and purified from several seed sources (McCleary *et al.*, 1981; McCleary, 1983).

Studies by McCleary and co-workers have shown that purified legume α-galactosidases act in the expected manner on guar galactomannan and lead to depletion of the galactose content of the polysaccharide without inducing any backbone scission (McCleary *et al.*, 1981, 1984*a*). Enzymically modified guar galactomannans containing similar galactose/mannose ratios to locust bean gum were found to exhibit similar physical properties, e.g. co-gelation with xanthan polysaccharide. It has therefore been established that α-galactosidase action on guar gum leads to the production of galactomannans with decreased galactose contents and increased functional value. However, two features of the protocol used in these studies (McCleary *et al.*, 1981, 1984*a*) were unsuitable for large-scale operation: Firstly, reactions were carried out in dilute (but viscous) solutions and pastes, leading to large reaction volumes and high water-removal costs; and secondly the α-galactosidase used was extracted in small amounts from germinating legume seeds and required extensive purification to eliminate contaminating depolymerisation enzyme activities (particularly endo-β-mannanase). Potential solutions to both of these problems have been disclosed in recent patent applications (McCleary *et al.*, 1984*b*; Overbeeke *et al.*, 1987). McCleary *et al.* (1984*b*) showed that effective α-galactosidase action could be achieved at guar gum concentrations up to 70% w/w, and Overbeeke *et al.* (1987) described the expression of guar α-galactosidase by a series of hosts transformed with recombinant DNA methods.

The present report describes a systematic study of the effect of guar gum concentration (and other important variables) on α-galactosidase

action, with the aim of identifying appropriate conditions for large-scale operation. Investigations into the properties of galactomannans produced by α-galactosidase treatment of guar gum are also described.

EXPERIMENTAL

Materials

Commercial grade guar gum (Hercules) produced by milling de-husked guar endosperms was used in all experiments. A purified, high viscosity locust bean gum (Meyhall) was used in comparative testing experiments. α-Galactosidase was obtained either by extraction and purification from germinating guar seeds using the method of McCleary (1983) (α-galactosidase II) or following expression of the guar endosperm enzyme in *Saccharomyces cerevisiae* (Overbeeke *et al.*, 1987), kindly supplied by Dr N. Overbeeke, Unilever Research Laboratory, Vlaardingen, The Netherlands.

Purity of enzyme preparations was established by gel electrophoresis; no detectable (McCleary, 1978) endo-β-mannanase activity was found in any preparation.

Enzyme and reaction assays

α-Galactosidase was assayed by monitoring the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside (McCleary, 1982); 1 nkat is defined as the amount of enzyme that releases 1 nmol of *p*-nitrophenol at pH 4·5 and 40°C in 1 s. Enzyme quantities used in guar modification trials were determined by this assay method immediately prior to the experiment. For the same activity against *p*-nitrophenyl-α-D-galactopyranoside, enzyme preparations from guar endosperm and *S. cerevisiae* had identical activities against guar galactomannan.

To obtain the galactose/mannose ratio of a modified guar galactomannan, samples (typically 60 mg solids) were removed from the reaction mixture and α-galactosidase was inactivated by heating at 90°C for 10 min. Samples were dispersed in 10% sodium hydroxide solution (5 ml), water (20 ml) was added, and the mixture was shaken for 60 min, homogenised at room temperature and left for 15 min. This treatment rendered all polysaccharide soluble but did not cause any polymer degradation as judged by viscosity measurements on neutralised samples. The galactose/mannose ratio of the modified galactomannan was determined by assaying solutions for total carbohydrate content (i.e. galactomannan + galactose) using the anthrone/sulphuric acid method (Loewus, 1952) and the free galactose produced by the enzymic

reaction. Free galactose content was determined by adding the sample solution (0·2 ml) to 0·2 M Tris buffer (pH 8·6; 2·7 ml) followed by 1% w/v NAD solution (0·1 ml) and 0·25 unit β-galactose dehydrogenase (from *E. coli*; Sigma) in 0·2 M Tris buffer (pH 8·6; 0·05 ml). A solution made up as above with the omission of β-galactose dehydrogenase was used as the blank and 10–80 μg galactose samples were used as standards. Solutions were incubated for 1 h at 37°C and absorbance was measured at 340 nm immediately after incubation. The accuracy of this method of determining polymeric galactose/mannose ratios was checked by subjecting sample solutions to dialysis to remove free galactose, and determining the galactose/mannose ratio of the residual polymer either by gas–liquid chromatography following acid hydrolysis, reduction and acetylation (Albersheim *et al.*, 1967) or by complete hydrolysis with α-galactosidase and β-mannanase in dilute solution followed by β-galactose dehydrogenase and total carbohydrate assays (McCleary *et al.*, 1983).

Properties of α-galactosidase-modified guar galactomannan

The molecular weights of galactomannan samples were assessed from intrinsic viscosity values using the relationship given by Robinson *et al.* (1982). Viscosity values were measured for dilute (<0·2%) aqueous galactomannan solutions using a Contraves low-shear 30 rotational viscometer. Intrinsic viscosities were determined using both Huggins and Kramer extrapolations (Robinson *et al.*, 1982).

The ability of modified galactomannans to co-gel with κ-carrageenan was investigated by measuring gel failure properties in a compression test. Samples were prepared by hydrating solid polysaccharide mixtures with a little deionised water to form a paste, then slowly adding deionised water to a final polysaccharide concentration of 1% w/v, followed by heating at 80°C for 10 min. Hot solutions were poured into moulds, cooled to room temperature, and stored overnight at 4°C. Self-supporting gels were transferred to an Instron Universal testing machine (1122) at room temperature, compressed at a uniform rate (1 cm/min) and the resulting stress was measured as a function of percentage compression (strain). The yield stress and break strain values were determined from plots of stress against strain.

The performance of galactomannans as ice cream stabilisers was investigated using a standard test formulation. Ice cream blocks were stored on a grid at 18°C and the melting rate was determined by collecting liquid passing through the grid; the retention of block shape was assessed visually.

RESULTS

Effect of galactomannan concentration on α-galactosidase action

Studies by McCleary and co-workers have demonstrated the removal of galactosyl residues from guar galactomannan in dilute solutions (0·09% or 0·5% w/v) using α-galactosidase isolated from lucerne (McCleary *et al.*, 1981) or guar (McCleary *et al.*, 1984*a*) seeds. The dilute solution conditions used in these experiments were not considered appropriate to large-scale operation owing to the high process volumes needed and the potential costs of water removal. Further studies by McCleary *et al.* (1984*b*) demonstrated an effective reaction of α-galactosidase with guar galactomannan at much higher substrate concentrations (2–70%) than those used previously. In order to define preferred conditions for large-scale process operation, we have studied the effect of guar galactomannan concentration on α-galactosidase activity.

Figure 1 shows the galactose content of galactomannans produced by the enzymic reaction as a function of substrate concentration under defined conditions of time and enzyme/substrate ratio, and at three temperatures. The observed physical state of the reaction system is also shown as a function of guar concentration. The boundaries defining the various physical states are subjective but give important guidance when considering appropriate conditions for large-scale operation. In dilute solution guar gum is highly viscous; upon increasing guar concentration, viscosity increases until a paste with some resistance to flow is formed. Further increases in guar concentration lead to an adhesive, gel-like structure. At concentrations greater than 15–20% w/w, particulate semi-solid structures are observed which become less adhesive and more friable with increasing guar concentration.

For all reaction temperatures studied (35°C, 42°C, 50°C), increasing guar concentration leads to an increase in reaction efficiency (as reflected in lower galactose contents of galactomannan products) until a maximum is reached, after which efficiency decreases with increasing concentration (Fig. 1). Significant differences are observed, however, between the relative efficiency at 35°C, 42°C and 50°C as a function of guar concentration. Thus at 42°C the reaction is most efficient at ~ 10% w/w guar and is more efficient than at 50°C for guar concentrations up to ~ 25% w/w. Reaction at 50°C is maximised at 20–30% w/w guar and is more efficient than at 42°C for substrate concentrations greater than ~ 25% w/w (Fig. 1). At 35°C, maximum enzyme efficiency is seen at ~ 2% w/w guar, with reductions in efficiency compared with 42°C or 50°C at guar concentrations greater than 10% w/w (Fig. 1). The results shown in

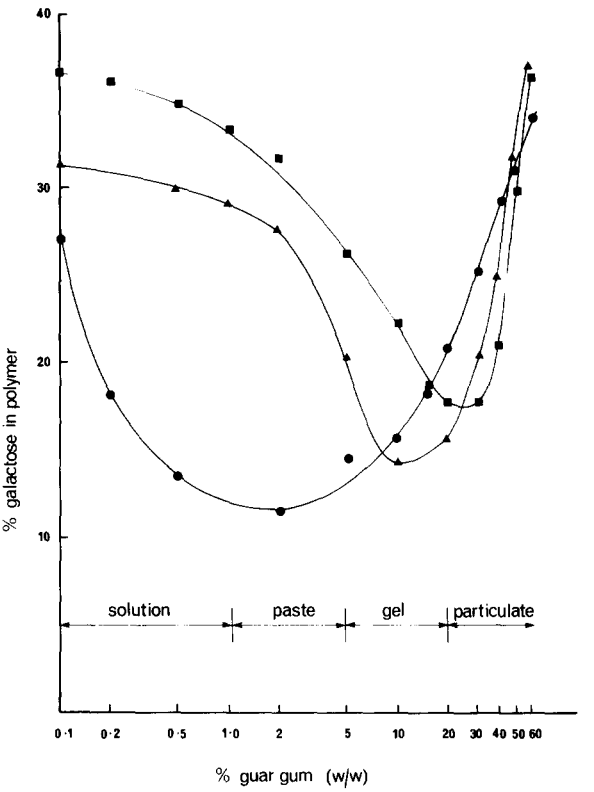

Fig. 1. The effect of galactomannan concentration on the reaction of α-galactosidase with guar gum. Reactions were carried out at 35°C (●), 42°C (▲) or 50°C (■) for 24 h at an enzyme loading of 370 nkat/g guar. Following reaction, the galactose content of modified galactomannans was calculated from measured values of free galactose and total carbohydrate. The physical state of the reaction mixture is also shown as a function of galactomannan concentration.

Fig. 1 demonstrate that considerable increases in reaction efficiency can be achieved by operating at higher guar concentrations than those used in most previous studies (< 1%) (McCleary *et al.*, 1981, 1984*a*) and, furthermore, that reaction systems such as semi-solid particulates can be utilised, with consequent improvements in processability and decreases in reaction volume compared with dilute viscous solutions. The mechanistic basis for the dependence of reaction efficiency on substrate concentration will be investigated in future studies. One inference which can be drawn from the present study, however, is that the reduction in reaction efficiency at very high (> 40% w/w) guar concentration is not due to irreversible enzyme inactivation. Thus, following partial reaction (7 h) with 180 nkat α-galactosidase per gram of guar at 50% w/w guar to give

a galactomannan containing 33% galactose, the reaction mixture was diluted to 30% w/w guar and the subsequent rate of galactose removal was found to be essentially identical to that observed for normal 30% w/w incubations. The effects of temperature on reaction efficiency (Fig. 1) will be discussed below.

It is interesting that the concentration of galactomannan in germinating guar seeds (where α-galactosidase activity occurs) is ~16% w/v (McCleary, 1983), i.e. in the concentration range where the most efficient α-galactosidase reaction at 42°C is observed (Fig. 1). This suggests the possibility that guar α-galactosidase may be structurally tailored to possess optimal activity against its natural substrate at physiological polymer concentrations.

Effect of temperature, time and enzyme level on α-galactosidase modification of guar galactomannan

We have noted that reaction temperature has interesting effects on the efficiency of α-galactosidase action as a function of guar galactomannan concentration (Fig. 1). These effects probably reflect variations in enzyme stability with substrate concentration. In dilute solution, guar α-galactosidase has been found to display optimal activity against the artificial substrate *p*-nitrophenyl-α-D-galactoside at 45°C, but on extended incubation the enzyme was found to be unstable above 40°C (McCleary, 1983). We have confirmed this finding and have furthermore found that enzyme activity in 30% or 40% w/w galactomannan systems is less sensitive to temperature than in dilute solutions, with significant inactivation occurring only at 55°C or higher. Thus, at 50°C enzyme activity is rapidly lost (<1 h) in dilute solutions, but is maintained for more than 24 h in particulate semi-solid systems. The greater efficiency of galactose depletion from solutions and pastes of guar gum at 35°C compared with 42°C, and at 42°C when compared with 50°C (Fig. 1), presumably reflects the greater stability of α-galactosidase at lower temperatures in these relatively dilute systems. With increasing concentration, temperature stability of the enzyme increases until, at $>25\%$ w/w galactomannan, reaction efficiency is greatest at 50°C due to standard kinetic effects. It appears that guar α-galactosidase experiences a rise in temperature stability of ~10°C between dilute solution/pastes and the semi-solid particulate state of its substrate. This could be due to a specific stabilising enzyme–substrate interaction at high concentrations or may reflect a general stabilising influence of the highly entangled polymeric matrix. Further studies are needed to investigate these possible mechanisms.

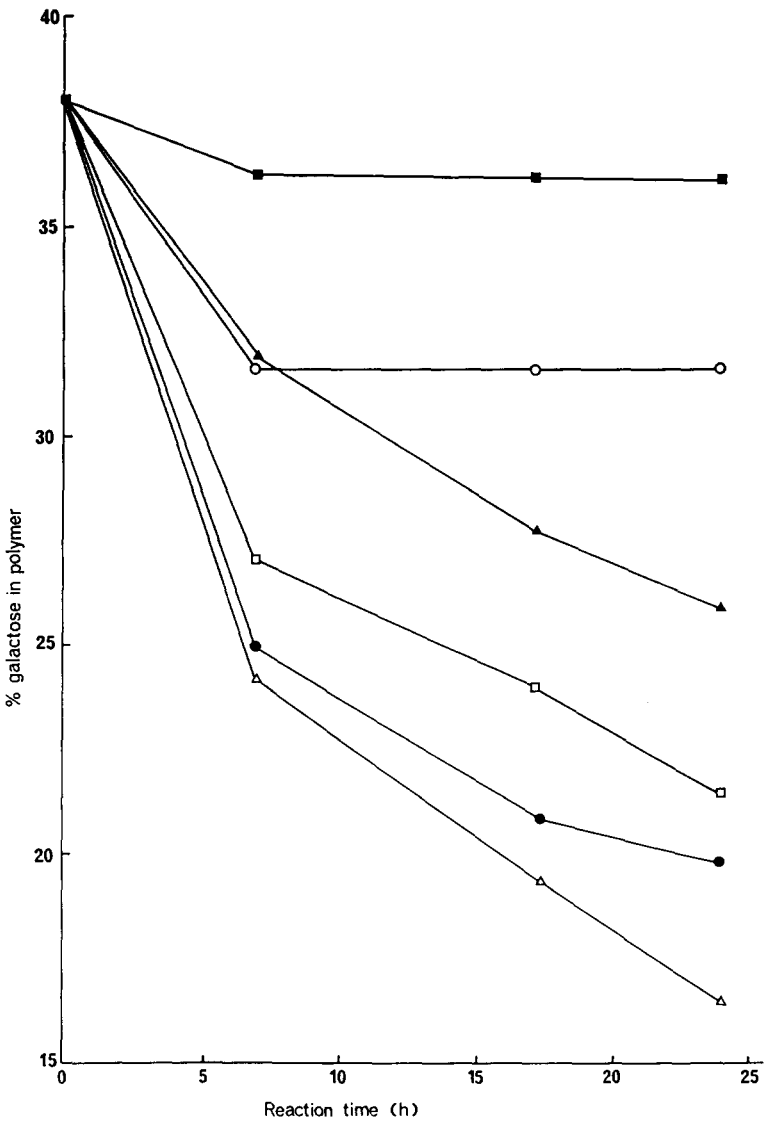

Fig. 2. The effect of reaction temperature on the enzymic depletion of galactose content from 30% w/w guar galactomannan at pH 4·5 and an enzyme loading of 370 nkat/g guar. Samples were withdrawn from reaction mixtures at the times shown, and polymeric galactose content was determined from assays for free galactose and total carbohydrate. The temperatures studied were 31°C (▲), 40°C (□), 47°C (△), 55°C (●), 62°C (○) and 70°C (■). Reaction profiles at 45°C and 50°C were essentially identical to that at 47°C (△).

As the semi-solid particulate reaction system (Fig. 1) was considered to be the most promising for large-scale operation, further experiments were carried out to assess the effects of temperature, time, pH and enzyme loading on the reaction of α-galactosidase with high concentrations of guar galactomannan. The optimum reaction pH was found to be 4·5–5·0 at 30% w/w guar concentration, identical to the optimum found for α-galactosidase-mediated hydrolysis of *p*-nitrophenyl-α-D-galactoside in dilute solution (McCleary, 1983).

Figure 2 shows the effect of temperature on the time course of reaction at 30% w/w guar concentration. The optimum temperature is found to be 45–50°C (reaction profiles at 45°C, 47°C and 50°C are essentially identical). At 55°C and above, significant irreversible enzyme inactivation is indicated by the observation of little or no change in polymer galactose content between 7 h and 17 h reaction times (Fig. 2). In other experiments we have established that enzyme activity is rapidly (< 2 min) destroyed at 90°C. At temperatures lower than 45–50°C, enzyme activity continues throughout the time scales studied but is significantly slower (Fig. 2) as would be expected for a kinetically controlled reaction.

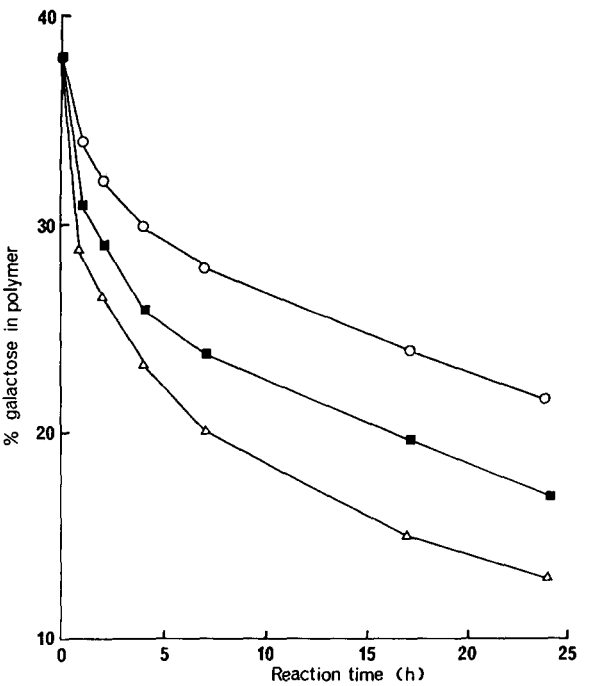

Fig. 3. The effect of enzyme/substrate ratio on the removal of galactose from 30% w/w guar galactomannan with α-galactosidase at 50°C and pH 4·5. Enzyme loadings were 170 nkat/g (○), 330 nkat/g (■) and 500 nkat/g (△) guar galactomannan.

Figure 3 shows the effect of enzyme/substrate ratio on the time course of galactose removal from 30% w/w guar galactomannan at 50°C. As would be expected, increased enzyme loading leads to more rapid depletion of galactose levels. Similar variations of galactose depletion rates with enzyme loading are found for 40% w/w guar reaction systems. In practical terms, various combinations of guar concentration, reaction time, and enzyme loading can be used to produce galactomannans with any desired galactose level. For large-scale production of any particular galactomannan type, the optimal conditions would presumably be determined by considerations of economy and the relative ease of processing.

Properties of α-galactosidase-modified galactomannans

Galactomannans produced by the action of α-galactosidase on guar gum in the semi-solid particulate concentration range have been characterised in terms of their chemical compositions (galactose/mannose ratio; see above), molecular weight, and functional performance in applications which currently utilise locust bean gum.

Molecular weights were characterised by intrinsic viscosity measurements and were found to be similar or slightly lower than for the starting guar galactomannan. On the basis of the relationship between intrinsic viscosity and molecular weight of guar gum given by Robinson *et al.* (1982), molecular weights of α-galactosidase-modified guar gum samples were found to be 5×10^5–2×10^6. Unmodified guar gum samples were estimated to have molecular weights of 2–3×10^6 by the same method. Modified guar gums prepared in solution and isolated by lyophilisation had similar intrinsic viscosity values to the starting material. Following reaction in the semi-solid concentration range, dried products required milling before complete solubility could be achieved. Some samples thus prepared had lower intrinsic viscosity values (corresponding to molecular weights of 5×10^5–1×10^6) than guar gum whereas others had similar values to the starting material. The slight depolymerisation sometimes observed was ascribed to the heat and friction generated by the laboratory hammer mill that was used to powder dried reaction products. The molecular weights of the modified galactomannans obtained are similar to those found for locust bean gum, and thus comparisons between the functional properties of enzymically modified guar gum and locust bean gum can be made.

One of the major industrial uses of locust bean gum is as a co-gelling agent with other polysaccharides, e.g. carrageenan, agar and xanthan (Dea & Morrison, 1975). The addition of locust bean gum to these

materials leads to significant increases in gel strength and, particularly for carrageenan, the production of a desirable elastic texture. Previous work on the interaction of xanthan gum with α-galactosidase-modified guar gum showed that the rheological properties of xanthan/locust bean gum mixed gels could be approximated by using modified galactomannans with galactose contents of 19–25% (McCleary *et al.*, 1981, 1984*a*). In the present investigation, we have studied the interaction of both locust bean gum and enzymically modified guar gum with κ-carrageenan through rheological failure testing of co-gels. The failure properties of a series of gels are presented in Table 1 from which it can be seen that, in co-gels with carrageenan, both the strength (yield stress) and elasticity (yield strain) imparted by locust bean gum are matched by α-galactosidase-modified guar samples of similar galactose contents. Compared with locust bean gum, modified guar gums of higher galactose content (26%, 28%) give weaker and less elastic gels, whereas for material of 20% galactose content a stronger but equally elastic gel is formed (Table 1).

A major application of locust bean gum is in the stabilisation of ice cream formulations. To examine the effectiveness of replacing locust bean gum with α-galactosidase-modified guar gum in ice cream applications, the stability properties of ice creams incorporating locust bean gum, guar gum, and modified guar gum (22% galactose) have been examined. Replacement of locust bean gum with guar gum led to more rapid ambient melting rates and loss of shape in an ice cream block, whereas an equivalent concentration of α-galactosidase-modified guar gum led to very similar ambient stability and shape retention to that imparted by locust bean gum.

TABLE 1

Effect of Galactomannan Galactose Content on Gelation Behaviour with κ-Carrageenan[a]

Galactomannan type	*% Galactose*	*Yield stress (Pa)*	*Break strain (%)*
Modified guar gum	28	5 400	45
Modified guar gum	26	15 600	67
Modified guar gum	24	21 500	76
Modified guar gum	22	23 000	73
Modified guar gum	20	39 500	77
Locust bean gum	23	21 700	75

[a]0·5% galactomannan + 0·5% carrageenan. Neither 1% carrageenan nor 0·5% carrageenan + 0·5% guar formed self-supporting gels under the conditions used.

DISCUSSION

There are two galactomannans that are currently available in commercial quantities, guar gum and locust bean gum. In certain applications (e.g. co-gelation with carrageenan and stabilisation of ice cream), locust bean gum offers unique functional properties and is the ingredient of choice. The price of locust bean gum has historically been higher than that of guar gum and is likely to remain so. The relative ability of the two materials to respond to market demands is also different since locust bean gum is obtained from a perennial tree and production levels cannot be increased rapidly, whereas guar is an annual cultivated plant. Furthermore, guar plants thrive in semi-arid conditions with little competition from other crops, whereas carob (locust bean) trees are grown in Mediterranean countries with competition for land from other cash crops and tourism. These factors suggest that the stability of supply and economy of guar gum is likely to remain superior to that of locust bean gum in the future.

Against this background it is of commercial interest to determine whether the modification of guar gum with α-galactosidase can be used to produce economically a substitute for locust bean gum. This report has shown that high enzyme activity can be achieved in semi-solid reaction systems thereby minimising process volumes and dewatering costs. The production of active enzyme in a manner amenable to industrial scale-up has also been achieved and will be described elsewhere. The optimum process operating conditions will depend on preferred processing routes and economy of operation, and could be achieved by varying a number of factors, particularly substrate concentration, enzyme/substrate ratio, reaction temperature and time.

Although the application of α-galactosidase-modified guar galactomannan as a substitute for locust bean gum is attractive in itself, the technology described allows for the production of galactomannans with a wide range of galactose contents. It is possible that some of these materials may have additional unique properties and offer novel functional performance in foods and other applications.

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