ELSEVIER

Carbohydrate Research 282 (1996) 247–262

CARBOHYDRATE RESEARCH

The chemical structure of amylose and amylopectin fractions of starch from tobacco leaves during development and diurnally–nocturnally

Norman K. Matheson

Department of Agricultural Chemistry and Soil Science, University of Sydney, Sydney NSW 2006, Australia

Received 11 July 1995; accepted 14 November 1995

Abstract

Starches, isolated from rapidly expanding tobacco leaves four times during the day and night and once from fully expanded leaves, were fractionated with concanavalin A. From an examination of the amounts and properties of amylose, the diurnal decrease in iodine absorption of the starches on illumination appeared to be due to an increase in its branched character, and possibly the presence of unbranched polymer of low dp, combined with a decrease in the proportion of amylose fraction. The increase in apparent amylose content with age was due to an increase in the proportion of amylose. The amylose fractions at different times had only small differences in average mol size in solution and relative mol wt (M_r near 4×10^5) which were lower than those of storage organs. The average mol size in solution and relative mol wt of the amylopectins decreased during illumination, increased in darkness, and were lower (M_r $2–2.7 \times 10^6$) at all times than those from storage organs. Debranching followed by size-exclusion chromatography [on Fractogel TSK 50(S)] gave similar proportions of long, medium, and short chains for all amylopectin samples, and these proportions differed from those for debranched amylopectin from *n*-maize seed starch. On debranching and chromatography of the amylopectin β-limit dextrins (which gives an estimate of the proportions of core chains) differences persisted. Structural characteristics of amylopectin from tobacco leaf starch were similar to those of normal genotypes from storage organs. The proportion of glucosyl units in core chains, the external-to-core chain ratio, and indices of compactness were calculated for a number of (1 → 4)(1 → 6) α-glucans. A plot of the index of compactness for glycogens and amylopectins showed that the decrease in compactness and the increase in total average chain length that occurs from glycogen to normal and then to amylose extender amylopectins involves a proportionate increase in average internal, external, and core chain lengths and not a selective increase in one type of chain.

Keywords: Amylose; Amylopectins; Tobacco leaves; Branching; Starch

SSDI 0008-6215(95)00381-9

1. Introduction

There have been extensive studies on chemical structural aspects [1] of storage starches in seeds, such as cereals [2–5] and peas [6], as well as tubers, e.g., potatoes [7], but fewer on leaf starches. In leaves, starch is metabolically more mobile, although in tobacco a pattern of storage during leaf development has also been found [8]. As starch accumulates in seeds and tubers, its isopotential iodine absorption increases, and this has been related to an increase in the proportion of the amylose component [1,9,10] as it binds iodine tightly. In tobacco leaves, as the starch content increases during maturing of the leaves, the iodine absorption increases [8]. Unlike glycogen, starch in plants also contains a relatively unbranched $\alpha(1 \rightarrow 4)$ glucan (amylose). The relationship between the two polymers during starch biosynthesis and degradation is not clear. In contrast to storage organs, from which starch is usually examined, in leaves rapid biosynthesis and depletion takes place. In tobacco leaves large changes in starch content occur diurnally and during development [8,11].

Variation in iodine absorption also occurs in tobacco [11–13] and cotton leaves [14] on a diurnal–nocturnal basis. In mature tobacco leaves [12] starch–iodine spectra indicated that the proportion of apparent amylose was higher in the day than at night. However, two other studies with tobacco leaves [11,13] have shown the inverse pattern. In rapidly expanding leaves and seedling leaves [11] a potentiometric estimation of isopotential iodine absorption indicated that the proportion of apparent amylose decreased during the day and increased at night. In leaves that had just expanded to full size [13] a similar result was obtained when the amylose–amylopectin ratio was estimated by a paper chromatographic method. In cotton leaves [14], illuminated during a ten-hour period, starch accumulation was inversely related to the apparent amylose–amylopectin ratio when estimated colorimetrically after iodine addition. Iodine spectra of older leaves indicated an increasing resistance to degradation of amylopectin in the dark [15]. When isolated chloroplasts from bean leaves [16] were illuminated and the amylose–amylopectin ratio measured by size-exclusion chromatography on Sepharose 4B, the proportion of amylose varied with time of illumination. Whereas in senescing tobacco leaves (both attached to [17] and detached from [18] the plant) no change in the amylose–amylopectin ratio accompanied depletion, in cotton leaves [15] changes were observed. The different patterns found for apparent amylose contents in leaves [11–18] indicates that whereas the apparent amylose content varies, factors other than time of sampling may be operating.

The effective preparative fractionation of tobacco leaf starches, collected from rapidly expanding tobacco leaves at various times of the day and night, and also when they were fully mature, as well as the structural changes that these fractions undergo are now reported. The properties described include various average chain lengths, chain length distributions, A:B chain ratios, average frequencies of substitution and relative average molecular sizes in solution and mol wt of the amylopectins, and iodine absorptions, relative average molecular sizes in solution and weights of the amyloses.

2. Results and discussion

Isolation and fractionation of starch.—Rapidly expanding leaves were sampled at dawn, 11 a.m., dusk and 11 p.m. and fully expanded leaves at 11 a.m. Starch was isolated as granules.

When fractionation was attempted by complexing with *n*-butanol, the isopotential iodine absorption of the precipitated fraction was much lower than expected for amylose and that of the soluble fraction was much higher than expected for amylopectin, indicative of possible extensive cross-contamination. Repetition of complexing did not substantially alter the iodine values. The problem was not due to incomplete dissolution of the starch, since centrifugation at 14,000*g* prior to addition of *n*-butanol gave no precipitate. It was also not due to a diffuse boundary between the *n*-butanol complex and the supernatant, as is found with cereal samples, since centrifugation of the *n*-butanol complex gave a sharp boundary. The amylose and amylopectin fractions were also not effectively separated by size-exclusion chromatography. When the starch samples were chromatographed, the amounts of the lower molecular weight portion differed from the values expected from iodine absorption and the wavelength maximum was much lower than expected for amylose. These effects were more pronounced in samples from rapidly expanding leaves. Incomplete fractionation on complexing with *n*-butanol occurs with amylose extender starches, as these contain both amylose and amylopectin of low molecular weight, and the longer chains of its amylopectin may complex. The solution after complexing contains low molecular weight amylose [19] and the precipitate some amylopectin. Cross-contamination has also been detected in the fractions from a number of normal starches, such as wheat, pea, potato, maize, and bean [9,20–22]. To overcome these problems, in some studies [23,24] an additional step such as ultracentrifugation [9,25,26] or size-exclusion chromatography [9,27,28] has been applied. This is effective with normal but not with high amylose starches. But each additional fractionation procedure increases the risk of hydrolysis of glycosidic linkages and a further complication is the tendency for amylose to retrograde on standing, particularly as it is purified. Retrograded amylose is very difficult to re-dissolve. The presence of amylose in an amylopectin fraction leads to incorrect values for its parameters.

Precipitation of the branched fraction by a high concentration of concanavalin A provides a method for efficient fractionation comprising a single step, and which can be used analytically [29] or preparatively [20,30]. This method [29] was applied for quantitative estimation of amylose content (Table 1). The absorption (A_{max}) per unit weight of glucan at the absorption maximum (λ_{max}) was also measured. The λ_{max} for all samples was 620 nm as expected for amylose. The amylose content in rapidly expanding leaves was lower during the day and there was an increase in amylose content with time of development. The values for 100 A_{max} μg^{-1} of glucan varied diurnally with lower values during the day. They were all lower than those for the amyloses from *n*-maize and *n*-rice starches. The starch samples were then fractionated with concanavalin A on a preparative scale [20,30].

Properties of amylose fractions.—The soluble fractions (amylose) were examined by size-exclusion chromatography on Sepharose CL-4B, and the glucan contents and

Table 1
Estimation of amylose content of tobacco leaf starches with concanavalin A

Time of sampling	Amylose (%) (±0.4)	λ_{max} (nm)	$100A_{max}$ /concn amylose (μg/mL) (±0.03)
Rapidly expanding at dawn	17.2	620	1.78
Rapidly expanding at 11 a.m.	15.7	620	1.74
Rapidly expanding at dusk	16.1	620	1.65
Rapidly expanding at 11 p.m.	16.7	620	1.72
Fully expanded at 11 a.m.	20.3	620	1.78

spectra of the iodine complexes of the column fractions were determined. The elution patterns for samples from rapidly expanding leaves at 11 a.m. and fully expanded leaves are shown in Fig. 1A and 1C. No material of higher molecular size with λ_{max} and A_{max} per unit weight values corresponding to amylopectin was detected by chromatography on Sepharose CL-2B, indicating efficient fractionation. The elution behaviour, λ_{max} and 100 A_{max} per unit weight of glucan for the five samples from tobacco leaves and from

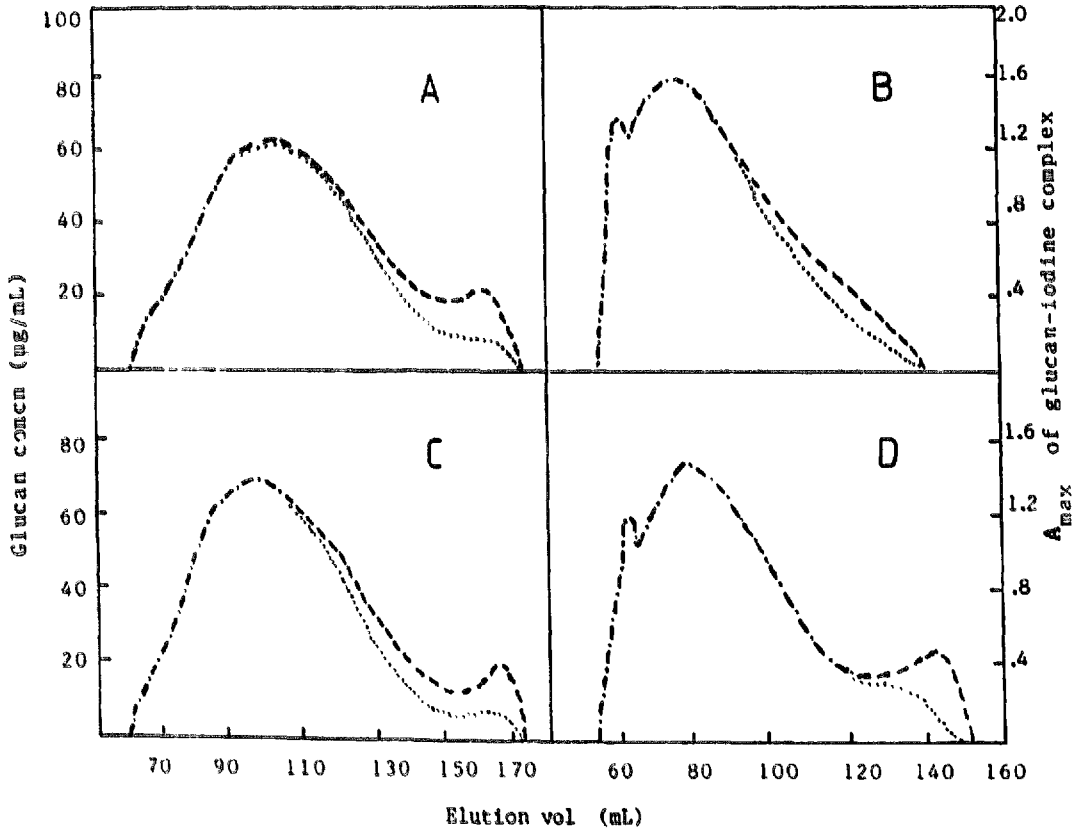

Fig. 1. Size-exclusion chromatography of amylose fractions from tobacco leaves: (A) rapidly expanding (11 a.m.) on Sepharose CL-4B; (B) rapidly expanding (11 a.m.) on Sepharose CL-6B; (C) fully expanded on Sepharose CL-4B; (D) debranched rapidly expanding (11 a.m.) on Sepharose CL-6B. ••• Glucan concentration; · · · A_{max} of the glucan–iodine complex.

Table 2
Size-exclusion chromatography of amylose and amylopectin samples

Glucan and column matrix	Source	$-\log K_{AV}$	λ_{max} (nm)	Average $100 A_{max}$ /concn glucan (μg/mL)
Amylose on Sepharose CL-4B	Tobacco leaves			
	Rapidly expanding			
	dawn	0.35	620(630) [a]	1.8
	11 a.m.	0.35	620(630) [a]	1.7
	dusk	0.37	620(630) [a]	1.7
	11 p.m.	0.36	620(630) [a]	1.7
	Fully expanded	0.38	620, 630	1.8
	n-Pea seeds [b]	0.59	630, 640	2.1
	n-Maize seeds [c]	0.59	620, 630	1.9
Amylopectin on Fractogel TSK 75(S)	Tobacco leaves			
	Rapidly expanding			
	dawn	0.48	560	1.0
	11 a.m.	0.42	560	1.0
	dusk	0.27	560	1.0
	11 p.m.	0.39	560	1.0
	Fully expanded	0.36	550–560	1.0
	n-Pea seeds [b]	0.69	550	0.9
	n-Maize seeds [c]	0.66	540(550)	0.9

[a] For major part of elution profile.
[b] From ref. [20].
[c] From ref. [10].

n-pea and *n*-maize seeds are given in Table 2. Chromatography of the rapidly expanding (11 a.m.) sample on Sepharose CL-6B (Fig. 1B) indicated that the second glucan peak appearing on CL-4B was due to foreshortening of the elution pattern because of the presence of low molecular weight material in a single molecular-size distribution and not a second distribution, since on CL-6B the second elution peak present on CL-4B became part of the tail of the main peak. The $-\log K_{AV}$ values (Table 2) for all tobacco samples were close, indicating only minor differences in average molecular size in solution. The $-\log K_{AV}$ value is directly proportional to molecular size in solution. All values were lower than those from the seed storage organs of *n*-maize and pea.

The λ_{max} values (Table 2) were in the range of that expected for amylose. The 100 A_{max} μg^{-1} glucan values were similar to those obtained in the estimation of amylose content and were slightly lower than those obtained for *n*-pea and *n*-maize amyloses. The values for tobacco were less in the lower molecular size range of the elution profiles, and the λ_{max} values were also slightly lower in the latter part of this region. The A_{max} per unit weight and the λ_{max} values both decrease as the chain length of an unbranched $\alpha(1 \rightarrow 4)$-linked glucan chain decreases, or when $\alpha(1 \rightarrow 6)$ branching is introduced [31–34], indicating that the amylose fractions contained branched material or some short $\alpha(1 \rightarrow 4)$ chains. Comparison of the elution profiles on Sepharose CL-6B before and after debranching of amylose from rapidly expanding leaves showed (Fig. 1B and 1D) a significant increase in chains of lower molecular size. The $-\log K_{AV}$ value

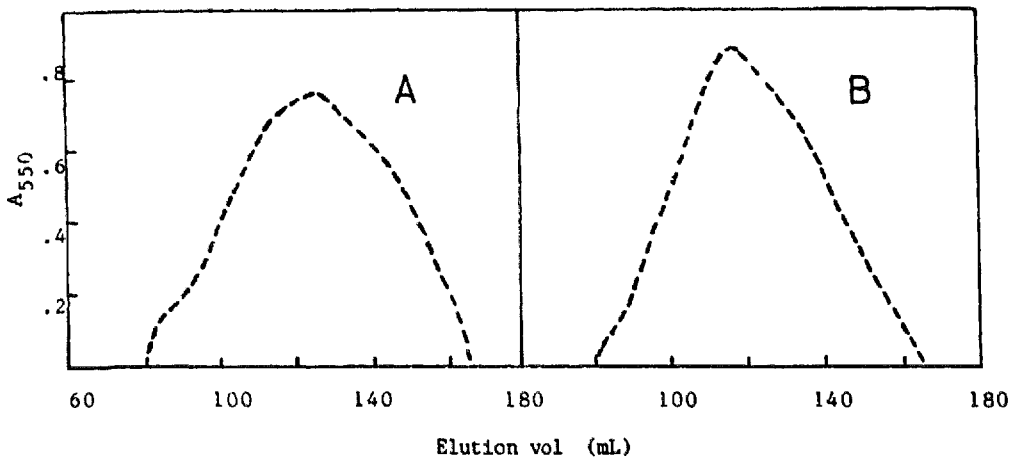

Fig. 2. Size-exclusion chromatography of periodate oxidised-borohydride reduced amyloses on Sephacryl S-400: (A) from rapidly expanding tobacco leaves (11 a.m.); (B) from fully expanded tobacco leaves.

decreased from 0.49 to 0.41, indicating the presence in the original amylose of branched material of relatively low molecular weight in significant amounts. Short-chain unbranched chains may also have been present. Similar results were obtained with the other amylose fractions from rapidly expanding leaves and fully expanded leaves.

The average M_r (molecular weight relative to protein standards) was estimated for amylose fractions from rapidly expanding leaves after oxidation by periodate and reduction with borohydride [35]. Periodate oxidation converts the repetitively internally bridged structure of the $\alpha(1 \rightarrow 4)$ glucan, in which the atoms in the pyranose rings are unable to rotate, to a polymer in which these atoms can rotate. Borohydride reduction stabilises the bonds. Reaction causes a slight change in mol wt (+1%) but a significant decrease in molecular size in solution, consistent with a change from a somewhat extended conformation to a globular molecule, the mol wt of which can be compared by size-exclusion chromatography with standard globular proteins. The oxidised–reduced samples of amylose were chromatographed on Sephacryl S-400 against standard proteins. The elution profiles for oxidised–reduced amylose from rapidly expanding leaves at 11 a.m. and fully expanded leaves are shown in Fig. 2A and 2B. The $-\log K_{AV}$ values for rapidly expanding (11 a.m.) and fully expanded samples were 0.35 and 0.38, giving estimated average M_r values of 3.9×10^5 and 4.4×10^5, i.e. average dp values of 2.4×10^3 and 2.7×10^3, retaining the relationship in molecular size in solution found for the original (unoxidised) samples on Sepharose CL-4B (Table 2).

The results indicate that the decrease in iodine absorption of tobacco leaf starch during illumination was due to a decrease in the absorption of the amylose fraction, consistent with increased branching (and possibly the presence of unbranched glucan of low dp) combined with a decrease in the proportion of amylose fraction. However, substantial quantities of amylose were always present. Amylose is defined as that portion of the starch not precipitated by concanavalin A, with a λ_{max} at or near 620 nm, and which is of low mol wt. Mature leaves contained a higher proportion of amylose fraction than rapidly expanding leaves consistent with a storage function of leaves. The average molecular sizes in solution and relative mol wts of the amylose fractions

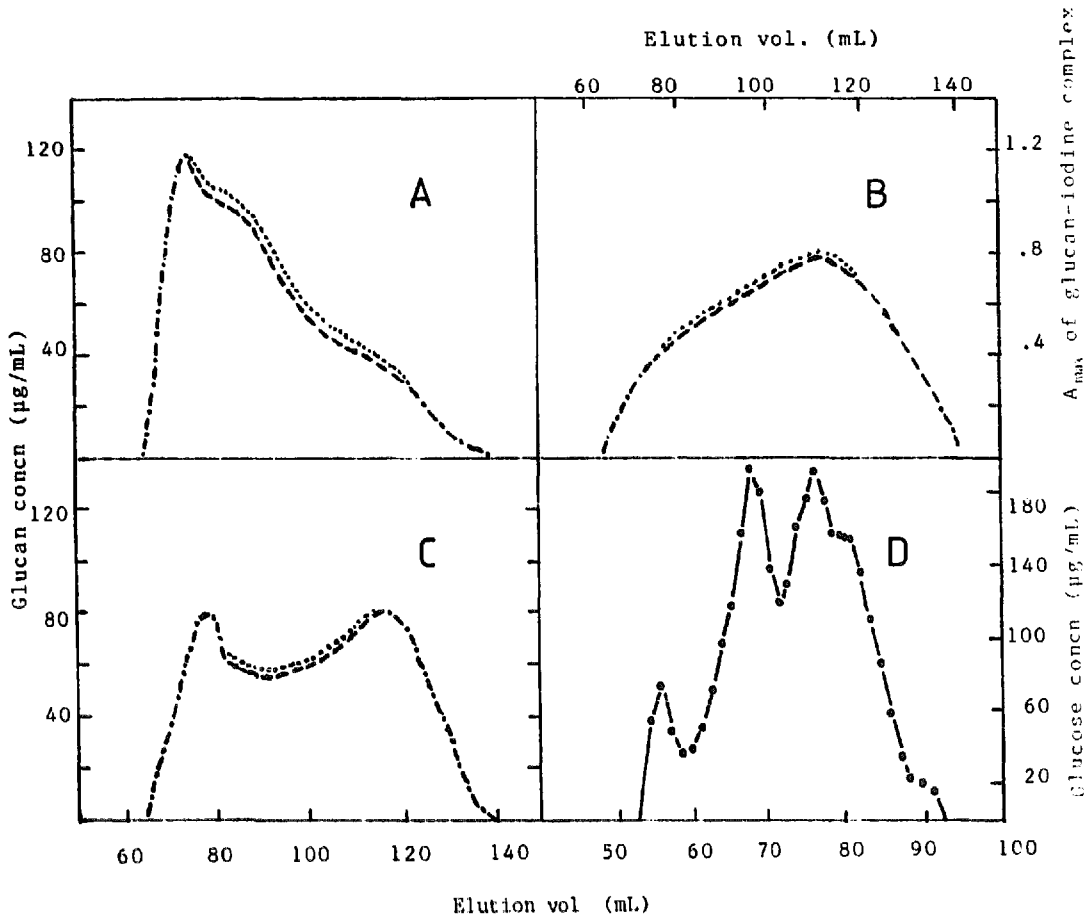

Fig. 3. Size-exclusion chromatography of amylopectin fractions from tobacco leaf starch: (A) rapidly expanding (dawn) on Fractogel TSK 75(S); (B) rapidly expanding (dusk) on Fractogel TSK 75(S); (C) fully expanded on Fractogel TSK 75(S); (D) debranched fully expanded on Fractogel TSK 50(S). --- or · · · · glucan concentration; · · · A_{max} of glucan–iodine complex.

sampled at all five times showed only small variations and were lower than those from seed storage organs (*n*-pea and *n*-maize seeds).

Properties of amylopectins.—The amylopectin fractions resulting from precipitation by concanavalin A were shown to be free of amylose by size-exclusion chromatography on Fractogel TSK 75(S) — the λ_{max} and $100A_{max}$ μg^{-1} glucan values (Table 2 and Fig. 3A, 3B, and 3C) were those of amylopectin throughout the whole elution profile of all samples. Chromatography on Sepharose 4B-CL and 6B-CL also indicated the absence of any amylose fraction (of low molecular size and high λ_{max}). Amylopectins from rapidly expanding leaves had a considerable range of molecular sizes in solution with $-\log K_{AV}$ values (Table 2, Fig. 3A and 3B) decreasing during the light period and increasing in the dark, indicating that material of lower size is preferentially depleted in darkness, and, that, on illumination, preferential accumulation of polysaccharide of lower size occurs. Amylopectin from mature leaves was disperse (Fig. 3C), and of intermediate size. All leaf samples were of lower size in solution than those of amylopectins from the storage starch of *n*-pea and *n*-maize seeds. Lower molecular

Table 3
Proportions of types of chain lengths of debranched amylopectins on size-exclusion chromatography [Fractogel TSK 50(S)]

Source	Percentages of fractions		
	Long	Medium	Short
Tobacco leaves			
Rapidly expanding			
dawn	9	35	56
11 a.m.	8	36	56
dusk	6	38	56
11 p.m.	7	36	57
Fully expanded	6	38	56
n-Maize seeds [a]	10	24	66
n-Pea seeds [b]	8	28	64

[a] From ref. [10].
[b] From ref. [20].

sizes in solution for amylopectins have been estimated by size-exclusion chromatography of this polymer from other leaf tissues, bean chloroplasts [16] and germinating lupin cotyledons [36].

Average relative mol wts were measured for the periodate oxidised, borohydride reduced β-limit dextrins by size-exclusion chromatography on Fractogel TSK 65(S) with protein standards [35]. Average M_r values for the amylopectins were then calculated after allowing for the loss of maltose on β-amylolysis. For the amylopectins from mature leaves and rapidly expanding leaves sampled at 11 a.m. and dusk, these were 2.5×10^6, 2.7×10^6, and 2.0×10^6 respectively, i.e. average dp values of 15×10^3, 17×10^3, and 12×10^3, lower than generally obtained for amylopectins from storage organs but approximately tenfold the values for the accompanying amylose. Despite the differences in mol wt of these samples the number of tiers of $\alpha(1 \rightarrow 4)$ chains (9 as the nearest whole number) were the same.

The structure of a biodendrimer like amylopectin can only be described in statistical terms, by parameters such as the averages of the various $\alpha(1 \rightarrow 4)$ glucan chain lengths, average mol wt, average branching frequency, and distributions of chain lengths. The last can be found by size-exclusion chromatography after debranching. When the tobacco amylopectins were debranched and chromatographed on Fractogel TSK 50(S) the chain length distributions were similar for all samples. The elution profile for amylopectin from rapidly expanding leaves at 11 a.m. is shown in Fig. 3D, and the proportions of long, medium, and short chains for all the samples in Table 3. Comparison with debranched *n*-pea and *n*-maize samples (Table 3) showed that the tobacco samples had a higher proportion of medium chains. Other than the differences in average molecular size in solution of the tobacco samples (Table 3 and Fig. 3A, 3B, and 3C) their molecular architecture appears to be similar at all stages of synthesis and depletion, suggesting that adequate levels of branching enzyme with the same branching pattern are present during synthesis and that the deposition of amylose and its increasing levels during development are not a consequence of limiting levels of this enzyme.

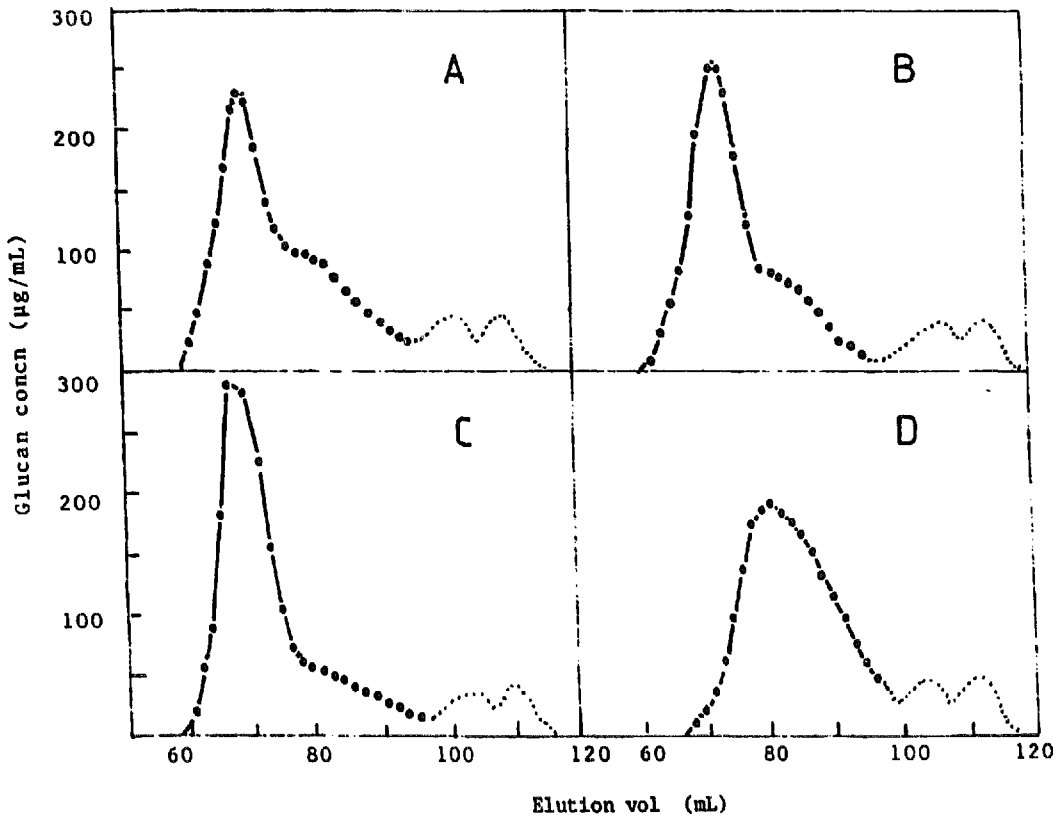

Fig. 4. Size-exclusion chromatography of debranched β-limit dextrins on Fractogel TSK 40(S) in series with TSK 50(S): (A) *n*-maize amylopectin; (B) amylopectin from rapidly expanding (11 a.m.) tobacco leaves; (C) *ae*-maize amylopectin; (D) rabbit liver glycogen. · · · Maltose and maltotriose fraction; · - · - · glucan of dp > 5.

The chain-length distributions of core chains of amylopectins from tobacco (rapidly expanding, 11 a.m.), *n*- and *ae*-maize amylopectins, as well as rabbit liver glycogen were then compared by debranching and chromatographing their β-limit dextrins. After debranching and size-exclusion chromatography on a column of Fractogel TSK 40(S) and 50(S) in sequence, the maltose and maltrotriose from the remnants of A chains could be sufficiently separated from the longer chains to give a distribution of the β-amylase-modified B chains. The profile (excluding the maltose-maltotriose section) is that of the core chains extended by 2 or 3 glucose units (in equal proportion). The elution profiles for the four debranched β-limit dextrins are shown in Fig. 4, in which the dotted section is material eluting at the same volumes as maltotriose and maltose. Comparing Fig. 4A and 4B, the tobacco polymer still has a higher proportion of its glucosyl units in longer core chains than *n*-maize. In all these profiles the ordinate is the amount of glucose in chains, not the number of chains [37]. The *ae*-maize sample (Fig. 4C) has an even higher proportion of glucose in longer than in shorter core chains, and the glycogen (Fig. 4D) the reverse. Chromatography on Sephadex G-50 of debranched β-limit dextrins prepared from whole starches of *w*-, *n*- and *ae*-maize has previously shown differences in their elution patterns [38].

Further properties of mature tobacco amylopectin and other (1 → 4)(1 → 6) α-glucans.—Detailed characteristics [average chain length (CL), degree of β-amylolysis, and A:B chain ratio] of the structure of the amylopectin from fully expanded leaves were then determined (Table 4) and compared with other (1 → 4)(1 → 6) α-glucans. Average core chain length (CCL), internal chain length (ICL), external chain length (ECL), fractions of A chains, and frequency of chain substitution were then calculated [39]. Comparison of the various structural parameters of tobacco leaf amylopectin (Table 4) with those of other starches (see ref. [39]) shows that the tobacco values are in the range of normal starches.

Three new parameters of the dendrimeric structure of (1 → 4)(1 → 6) α-glucans were also calculated — the ratio of external chains to core chains (E:C), the fraction of glucosyl units in core chains, and an index of compactness. If the total number of chains is T and a and b are the fractions of A and B chains, since each B chain consists of two sections, an external part, and another that is a core chain (plus the outermost 6-branching glucosyl unit), the ratio is

$$\text{E:C} = \frac{aT + bT}{b} = \frac{1}{b}$$

which is equal to F (the average frequency of branching). Then if e and c are the fractions of external and core chains, we have

$$e = \frac{\text{E:C}}{1 + \text{E:C}} \quad \text{and} \quad c = 1 - e$$

The fraction of glucosyl units in core chains is the number of units in core chains divided by the total number of units. The latter is the number of units in external chains plus the outermost 6-glucosyl branching unit, plus the units in core chains. Therefore the fraction of glucosyl units in core chains is

$$\frac{T \times b\text{CCL}}{T[a\text{ECL} + b(\text{ECL} + 1) + b\text{CCL}]} = \frac{b\text{CCL}}{\text{ECL}(a + b) + b\text{CCL} + b} = \frac{b\text{CCL}}{\text{ECL} + b\text{CCL} + b}$$

Alternatively, after calculation of the fraction of core chains (c) and external chains (e) this value can be found from the formula:

$$\frac{c\text{CCL}}{e\text{ECL} + c\text{CCL} + c}$$

It can also be calculated from the β-limit dextrin when the fraction of units in core chains in the original amylopectin is

$$\frac{T \times b\text{CCL}}{T[2.5a + 2.5b + b\text{CCL}]} \times (1 - \beta) = \frac{b\text{CCL}}{2.5(a + b) + b\text{CCL}} \times (1 - \beta) = \frac{b\text{CCL}}{2.5 + b\text{CCL}} \times (1 - \beta)$$

Table 4
Properties of (1 → 4)(1 → 6) α-glucans

Property	Glucan							
	Amylopectin from fully expanded tobacco leaves	Phytoglycogen [a]	Rabbit liver glycogen [a]	*w*-Maize [a]	*w*-Rice [a]	*n*-Maize [a]	*ae* / *w*-Maize [a]	*ae*-Maize [a]
Average chain length (CL)	26							
Average internal chain length (ICL)	6							
Average external chain length (ECL)	13							
Average core chain length (CCL)	14							
Fraction removed by β-amylolysis	0.55							
Fraction of A chains	0.53							
A:B chain ratio	1.1							
Average frequency of branching (F)	2.1							
Index of compactness	0.11	0.25	0.20	0.12	0.12	0.11	0.085	0.065
Average relative mol wt (M_r)	2.5×10^6							
Average degree of polymerisation (dp)	15×10^3							
Fraction of glucan in core chains	0.33	0.47	0.40	0.34	0.34	0.34	0.37	0.34
E:C ratio	2.1	1.8	1.7	2.3	2.3	2.2	2.2	2.3
Number of tiers	9							
Average number of α(1 → 4) glucan chains	770							

[a] See ref. [39] for chain lengths, fractions, and ratios not shown.

where β is the fraction of molecule removed by β-amylase. (2.5 is the average chain size of B chain stubs plus the outermost 6-glucosyl branching unit.)

A number of studies have described some $(1 \rightarrow 4)(1 \rightarrow 6)$ α-glucans as having more compact structures than others. A numerical basis has been decribed for indicating the degree of compactness [40]. This index has now been modified. The indices of compactness in Table 4 have been calculated from the formula:

$$\frac{c(F+1)}{e\mathrm{ECL}}$$

which derives from simplification of

$$\frac{(F+1)}{\mathrm{CCL}+1} \times \frac{c(\mathrm{CCL}+1)}{e\mathrm{ECL}}$$

The first fraction in the latter expression is the average degree of branching divided by the chain length of the core chain (over which branching occurs). In this calculation that part of a B chain external to the outermost $\alpha(1 \rightarrow 6)$ linkage is considered to be a branch, so that the average number of branches is $F + 1$: one is added to the CCL to include the glucosyl unit which provides the outermost branch point. The second fraction is the ratio of the number of glucosyl units in the core chains together with the outermost 6-linked glucosyl unit, divided by the number of glucosyl units in external chains. It expresses the effect on compactness of the length and number of external chains relative to core chains. The higher the value of $c(F+1)/e\mathrm{ECL}$ the more compact the dendrimer. It can be considered as a numerical index of compactness and differs from the previously published formula [40] in including the terms c and e, so that it expresses the proportion of glucosyl units exterior to the outermost branching point to those inside, instead of the ratio of average chain lengths of exterior and core chains.

$\dfrac{c(F+1)}{e\mathrm{ECL}}$ can also be expressed as $\dfrac{b(F+1)}{\mathrm{ECL}}$

In Table 4 the E:C ratios, the fraction of glucosyl units in core chains, and the index of compactness of a number of $(1 \rightarrow 4)(1 \rightarrow 6)$ α-glucans are shown; tobacco leaf starch from data in this paper and the remainder from data in ref. [39]. The E:C ratios reflect the average frequency of substitution of chains. The glycogen and phytoglycogen have higher proportions of glucosyl units in core chains than the amylopectins, and the degree of compactness decreases from the glycogens to waxy (maize and rice), then normal (maize and tobacco leaf), and then the amylose extender samples.

Amylopectins from different sources belong to a family of biodendrimers with varying structural characteristics. The differing sources can arise from among species, genotypes of a species or at stages in plant development. The structural characteristics of a particular amylopectin will then depend on the pattern of action of the endogenous branching enzyme (or enzymes). Chain length studies of glycogens from different sources indicate that these also differ [34], and the family of biodendrimers includes these. An aspect of the relationship of these polymers is shown in Fig. 5, the relationship of the various average chain lengths to the compactness. The decrease in compactness

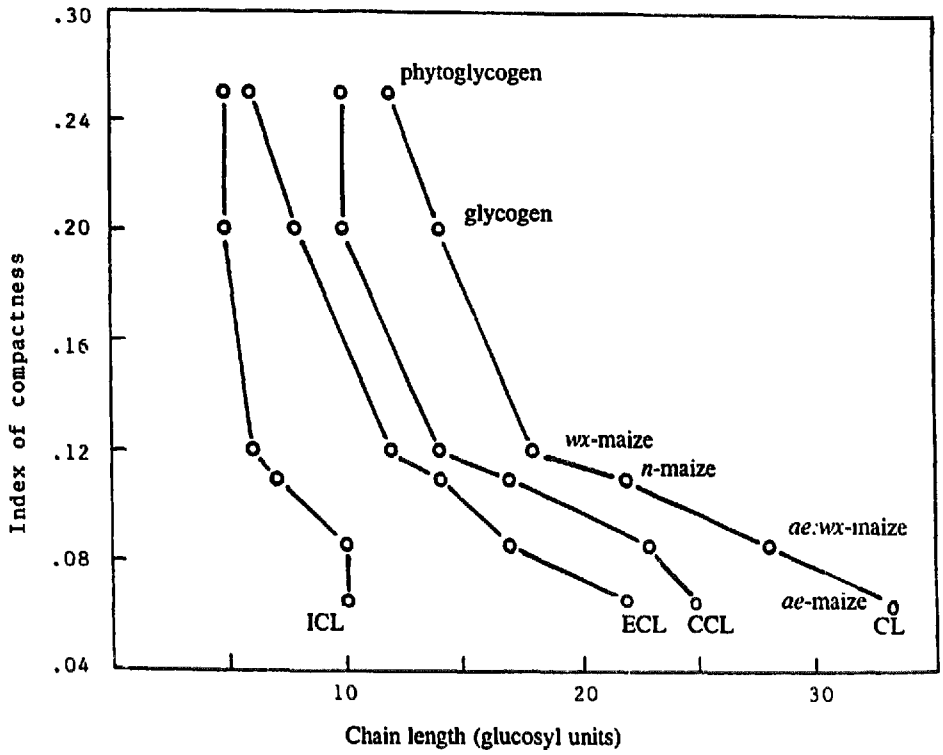

Fig. 5. Chain lengths of (1 → 4)(1 → 6) α-glucans vs. indices of compactness.

proceeding from glycogen to *ae*-amylopectin is associated with increases in the average chain lengths of all types of chains (external, core, interior, and total) in a relatively proportionate manner, and not with selective changes in one type of chain (e.g., exterior). This pattern leads to progressively more open molecules. The results are in accord with the earlier observations on chain length distributions of core chains (Fig. 4).

The occurrence of (1 → 4)(1 → 6) α-glucans with differing branching characteristics and average chain lengths raises the question of what factors produce these differences. One possible effect is branching enzymes with different substrate requirements in terms of the length of the α(1 → 4) glucan chain and the position of joining of the new α(1 → 6) linkage. Another is that decreased affinity of the branching enzyme for the α(1 → 4) glucan main chain may allow (on average) longer chains to form before branching occurs.

A preference for elongation by starch synthase of either A or B chains could lead to different patterns of dendrimeric structure. If A chains show a slight preference for elongation, on branching these by intra-chain transfer [41] (pathway A, Fig. 6), a polymer with an A:B chain ratio of less than one, with a compact spheroidal structure like glycogen, results. Exclusive branching of A chains with intra-chain transfer would lead to a laminated (Haworth) structure. Inter-chain transfer to another A chain would also reduce the A:B chain ratio, but that to a B chain would increase it. If B chains show a slight preference for elongation with intra-chain transfer, on branching (pathway B, Fig. 6) a polymer with an A:B chain ratio greater than one and an ellipsoidal shape like amylopectin is formed. Exclusive branching of B chains with intra-chain transfer would

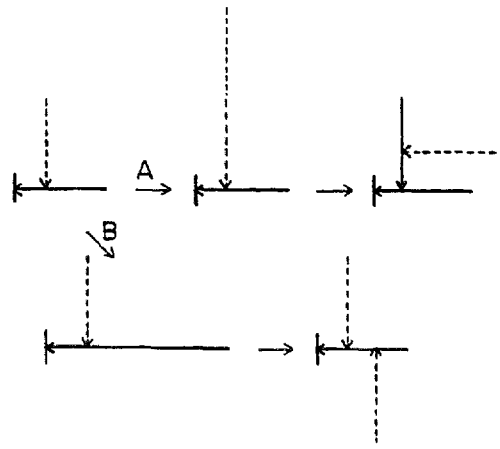

Fig. 6. Alternative intra-chain branching pathways: (A) by branching of A chains; (B) by branching of B chains. — B chain; - - - A chain; |← $\alpha(1 \rightarrow 6)$ branching point.

lead to a comb (Staudinger) structure. Inter-chain transfer to another B chain would also increase the A:B chain ratio, but that to an A chain would decrease it. If, on branching, the newly formed A chain and that section of the B chain external to the outermost branching point were of differing chain lengths and these chains associated (possibly as two helical sections), then the longer chain, with a non-associated non-reducing end, would react more readily with starch synthase, providing a mechanism for preferential extension of one chain.

3. Experimental

Plant sampling and isolation of starch granules.—The leaves of tobacco plants were numbered from the cotyledons as they appeared. Five adjacent leaves, that were known from a previous planting to become the largest on the plant, were used. When these leaves were in the rapidly expanding phase at dawn, 11 a.m., dusk, and 11 p.m., discs were cut from 60 plants from three positions on the one leaf, chosen randomly from the five labelled leaves. Discs were also cut when the leaves were fully expanded and the chlorophyll content had just started to decrease. Immediately after sampling, discs were macerated in a mixture of 0.1 M NaCl, 0.01 M $HgCl_2$, and toluene, the mixture passed through cheesecloth and the residue re-macerated and filtered until no more starch granules were produced. The granules were centrifuged (600 $g \times 20$ min, 20 °C) and purified by repeatedly shaking with toluene and salt solution, filtering through cheesecloth, and centrifuging. When white and free of cell debris, the purified granules were washed with ethanol, acetone, and ether and dried in a vacuum desiccator.

Dissolution and fractionation of granules.—Granules were dispersed in dimethyl sulfoxide and precipitated in ethanol and then fractionated as previously described [10,20,30,39].

Estimation of amylose content of starches.—The amylose content was estimated by reaction with phenol–sulfuric acid of an aliquot of the supernatant after concanavalin A precipitation of amylopectin [29]. Iodine spectra were measured on the same solution.

Preparation of β-limit dextrin and debranching.—These were performed by previously described methods [30,39].

Size-exclusion chromatography.—Chromatography of amylose on Sepharose CL-4B and CL-6B, amylopectin on Fractogel TSK HW 75(S), and of debranched amylopectin on Fractogel TSK HW 50(S) was performed following earlier methods [10,30,39]. Debranched β-limit dextrins were chromatographed on a column of TSK HW 40(S) (25 cm) in sequence with TSK HW 50(S) (63 cm) in a 1.4 cm diameter column.

Estimation of chain lengths and β-amylolysis limits.—The average total chain length of amylopectins was estimated from the reducing capacity with copper ions after debranching, the β-amylolysis limit by the reducing capacity with copper ions of released maltose, and the fraction of A chains by size-exclusion chromatography of debranched β-limit dextrin by previously described methods [20,39]. Average internal, external, and core chain lengths and average frequency of branching were calculated as described in ref. [39].

Determination of average relative mol wt (M_r) values.—To avoid the presence of ions in solution and the need to re-dissolve amylose, solutions for oxidation were prepared by twice washing with cold ethanol the amylose–iodine complex (ca. 5 mg) prepared from the supernatant left after precipitation with concanavalin A of the amylopectin in starch. The residue was suspended in water (2.0 mL), nitrogen was bubbled to remove oxygen and excess iodine, and the solution was warmed in a water bath (with nitrogen still passing) to remove bound iodine and dissolve the amylose. The previously described method [35] for determination of M_r was modified. After oxidation and destruction of excess periodate with dihydroxy butane the addition of lead acetate to precipitate iodate and the subsequent centrifugation step were omitted. Also, potassium borohydride was added without prior adjustment of the pH and then the centrifugation step was unnecessary. The changes gave a higher recovery of product. Polysaccharide (5 mg) in water (2.0 mL) was reacted with $NaIO_4$ (30 mg) in a gently stirred solution in the dark at 4 °C for 18 h. 2,3-Dihydroxy butane (20 μL) was added and after 2 h KBH_4 (5 mg) added and the solution was kept at room temperature for 4 h. Oxidised–reduced amylose was chromatographed on Sephacryl S-400 with β-amylase (2.0×10^5), apoferritin (4.43×10^5), and thyroglobulin (6.69×10^5) as standards. Oxidised–reduced amylopectin was chromatographed on Fractogel TSK HW 65(S) with apoferritin and thyroglobulin as standards. Since no standard protein with a mol wt as high as the β-limit dextrins was available, the mol wt $-\log K_{AV}$ relationship of the proteins was extended linearly. The amount of polymer in column fractions was estimated by heating with 2,4-dinitrophenylhydrazine followed by addition of ethanolic NaOH and measurement at A_{550}. All reagents, less polysaccharide, produced a small peak at the total volume of the column which was subtracted from the profiles obtained with samples containing polysaccharide.

References

[1] W. Banks and C.T. Greenwood, *Starch and its Components*, Edinburgh University Press, Edinburgh, 1975.

[2] C.W. Bice, M.M. MacMasters, and G.E. Hilbert, *Cereal Chem.*, 22 (1945) 463–476.

[3] M.J. Wolf, M.M. MacMasters, J.E. Hubbard, and C.E. Rist, *Cereal Chem.*, 25 (1948) 312–325.
[4] G. Harris and I.C. MacWilliam, *Cereal Chem.* 35 (1958) 82–83.
[5] M. Asaoka, K. Okuno, Y. Sugimoto, and H. Fuwa, *Agric. Biol. Chem.*, 49 (1985) 1973–1978.
[6] C.T. Greenwood and J. Thomson, *Biochem. J.*, 82 (1962) 156–164.
[7] R. Geddes, C.T. Greenwood, and S. Mackenzie, *Carbohydr. Res.*, 1 (1965) 71–82.
[8] N.K. Matheson and J.M. Wheatley, *Aust. J. Biol. Sci.*, 15 (1962) 445-458.
[9] N.K. Matheson, *Phytochemistry*, 10 (1971) 3213–3219.
[10] S-H. Yun and N.K. Matheson, *Carbohydr. Res.*, 227 (1992) 85–101.
[11] N.K. Matheson and J.M. Wheatley, *Aust. J. Biol. Sci.*, 16 (1963) 70–76.
[12] T. Mizuno, K. Katô, T. Fujita, and T. Kinpyô, *Bull. Agric. Shizuoka Univ.*, 10 (1960) 103–107.
[13] T. Kakie and Y. Sugizaki, *Soil Sci. Plant Nutrition*, 16 (1970) 201–203.
[14] C.W. Chang, *Plant Physiol.*, 63 (1979) 973–977.
[15] C.W. Chang, *Plant Physiol.*, 65 (1980) 844–847.
[16] M.I.P. Kovacs and R.D. Hill, *Phytochemistry*, 13 (1974) 1335–1339.
[17] I.R. Abbot and N.K. Matheson, *Phytochemistry*, 11 (1972) 1261–1272.
[18] T.P. Gaines and H.G. Cutler, *J. Agric. Food Chem.*, 22 (1974) 706–708.
[19] W. Banks and C.T. Greenwood, *Carbohydr. Res.*, 6 (1968) 241–244.
[20] N.K. Matheson, *Carbohydr. Res.*, 199 (1990) 195–205.
[21] R.W. Klingler and M. Zimbalski, *Starch*, 44 (1992) 414-418.
[22] C.G. Oates, *Starch*, 42 (1990) 464–467.
[23] T. Baba and Y. Arai, *Agric. Biol. Chem.*, 48 (1984) 1763–1775.
[24] Y. Takeda, S. Hizukuri, and B.O. Juliano, *Carbohydr. Res.*, 148 (1986) 299–308.
[25] H. Baum and G.A. Gilbert, *J. Colloid Sci.*, 11 (1956) 428–434.
[26] I.M. Savich and Y.V. Peruanskii, *Chem. Natural Compounds*, 14 (1978) 692–694 (translation of *Khim. Prir. Soedin.*).
[27] R. Ebermann and R. Schwartz, *Starch*, 27 (1975) 361–363.
[28] W. von Praznik, *Starch*, 38 (1986) 292–296.
[29] S.-H. Yun and N.K. Matheson, *Starch*, 42 (1990) 302–305.
[30] N.K. Matheson and L.A. Welsh, *Carbohydr. Res.*, 180 (1988) 301-313.
[31] J.M. Bailey and W.J. Whelan, *J. Biol. Chem.*, 236 (1961) 969–973.
[32] F.W. Fales, *Biopolymers*, 19 (1980) 1535–1542, 1543–1553.
[33] M. John, J. Schmidt, and H. Kneifel, *Carbohydr. Res.*, 119 (1983) 254–257.
[34] S.A.S. Craig, A.M.L. McDonald, D.J. Manners, and J.R. Stark, *Carbohydr. Res.*, 179 (1988) 327–340.
[35] L. Tao and N.K. Matheson, *Carbohydr. Polym.*, 20 (1993) 269–277.
[36] H.S. Saini and N.K. Matheson, *Phytochemistry*, 20 (1981) 641–645.
[37] T.N. Palmer, L.E. Macaskie, and K.K. Grewel, *Carbohydr. Res.*, 114 (1983) 338–342.
[38] C. Mercier, *Starch*, 25 (1973) 78–83.
[39] S.-H. Yun and N.K. Matheson, *Carbohydr. Res.*, 243 (1993) 307–321.
[40] S.-H. Yun and N.K. Matheson, *Proc. 43rd Aust. Cereal Chem. Conf.*, 1993, pp 59–62.
[41] D.J. Manners, *Carbohydr. Polym.*, 11 (1989) 87–112.