ELSEVIER

Journal of Chromatography A, 828 (1998) 515–521

JOURNAL OF CHROMATOGRAPHY A

Determination of the molecular mass of amylose

T. Suortti[a,*], M.V. Gorenstein[b], P. Roger[c]

[a]*VTT Biotechnology and Food Research, P.O. Box 1500, Fin-02044 VTT, Finland*
[b]*Waters Corporation, 34 Maple Street, Milford, MA 01757-3696, USA*
[c]*INRA, B.P. 71627, 44316 Nantes Cedex 03, France*

Abstract

Normally the reliable determination of the molecular mass of amylose is a very tedious procedure requiring several days of sample preparation to remove contaminating amylopectin. In the method presented the detection of amylose is based on its selective detection by post-column colourization after size-separation chromatographic separation. The quantification of amylose is based on totally linear synthetic amylose thus targeting the analysis on the most important quality of amylose, long linear chains. The molecular mass of amylose, which was the main target could be analyzed by very simple sample preparation. © 1998 Published by Elsevier Science B.V. All rights reserved.

Keywords: Molecular mass; Amylose; Carbohydrates; Starch; Amylopectin

1. Introduction

One of the Nature's most abundant polymers, starch, is composed of two main components; highly branched amylopectin (AP) and almost linear amylose (AM). Both contain glucose bound by α(1–4) linkages to form linear chains. In amylopectin most chains are rather short (average chain length ~20) and they are bound to longer chains by α(1–6)-bounds to form a highly branched structure which has a molecular mass in the range 10^9–10^7 depending on the botanical origin of the starch [1]. The amylose is composed of long linear glucose chains with α(1–4) linkages and its degree of polymerization (DP) is in a range of 300–10 000 again depending on its botanical origin. Although amylose is the minor component in most starches it has a major effect on the formation of viscoelastic pastes and gels in the cooling of heated amylopectin–amylose mixtures. These effects are strongly dependent on the molecular mass distribution [2,3].

Laser-light scattering offers a widely applied and reliable tool for determining the molecular mass of amylose, but it requires highly purified samples. Even slight contamination by amylopectin hinders reliable measurements because of the much higher molecular mass of this contamination. By combining laser-light scattering measurements with size-exclusion chromatography any contamination by amylopectin is easily distinguished [3] but size-exclusion chromatography systems are incapable of achieving the required resolution.

Tedious isolation procedures starting with defatting of the starch followed by dispersion in hot water and precipitation of amylose as a crystalline complex after the addition of suitable hydrophilic compounds such as 1-butanol [4] or thymol [5]. Several recrystallizations [6] or ultra-centrifugation [7] is required to effect recovery of pure amylose. All these methods depend on the ability of amylose to form helices with which these precipitating sub-

*Corresponding author.

0021-9673/98/$ – see front matter © 1998 Published by Elsevier Science B.V. All rights reserved.
PII: S0021-9673(98)00831-0

stances are complexed. The reverse is the purification of amylose by concanavalin A, which selectively precipitates amylopectin [8].

Because of its ability to form long helices, amylose can form highly coloured iodine complexes, As the chain lengths in amylopectin are much shorter, their ability to form complexes and bind iodine is much weaker. For a long time this has been used to determine the amylose content of starches by the spectrophotometric method [9] or by the potentiometric [10] or amperometric titration [11] of defatted samples.

The combination of size-exclusion chromatography analysis of starches with on-line [12] or off-line [13,14] iodine addition has been reported previously but a refinement of this technique to determine both amount and molecular mass of amylose after minimal sample preparation is reported here. Most natural (i.e. non-processed) starches can be analyzed directly by choosing the correct specific wavelength detection and the application of narrow-molecular-mass amylose standards. Also those molecules which have the structure between amylose and amylopectin (so-called intermediate fraction) with long enough linear chains and thus some amylose like qualities will be taken into account. For more complicated samples which contain by chromatography or by detectors specificity unresolved peaks, mathematical peak deconvolution can be applied.

2. Material and methods

2.1. Starch samples

Commercial rye and oat starch samples were obtained from Primalco, Rajamäki, Finland. The barley and potato starch was from the Raisio Group, Raisio, Finland. Wheat starch was from Cerestar, Brussels, Belgium. Waxy maize, ordinary maize and high amylose maize were from National Starch, NJ, USA.

These samples were dissolved by moistening a 200 mg sample with 5 ml of water for 1 h. Then, 5 ml of 2 *M* NaOH was added and stirring with slowly rotating magnetic stirrer was continued at least 6 h after which time the samples were diluted 1:10 with 1 *M* NaOH and injected without further manipulations. The whole sample preparation procedure was carried out under argon blanket.

Defatting of starch samples was done by dissolving 1 g starch in 20 ml 95% dimethyl sulfoxide (DMSO) by stirring for three days. Starch was then precipitated by addition of 5× volume of ethanol. The precipitate was recovered on a glass sinter and was washed with acetone and diethyl ether and dried. During sample dissolution oxygen care was taken to exclude oxygen by purging all solution with helium and working under an argon blanket.

2.2. Isolation of natural amylose fractions

Barley starch was leached at 95°C for 15 min. After cooling to room temperature the sample was centrifuged for 15 min at 3000 *g*. The solution was then centrifuged for 15 min at 40 000 *g* after an overnight cooling at 4°C. The precipitate from this second centrifugation was lyophilized and dissolved as a 0.5 g/l solution in 1 *M* NaOH. From this solution 40 parallel 150 μl injections were made in the SEC instrument described below, equipped with two μHydrolgel 2000 columns and 0.5 ml fractions were collected into tubes containing 0.5 ml of 200 m*M* pH 6.5 sodium phosphate buffer and 20 μl 1 *M* HCl. The parallel fractions were combined and centrifuged after precipitation at 4°C. The precipitates were washed with a small amount of cold water and lyophilized.

The dried fractions were dissolved in 1 *M* NaOH as ~1000 mg/l solution and their molecular mass and concentration were determined by SEC-laser light scattering and refractive index detection. The fractions were then used to construct both calibration curves for molecular mass determination and for concentration determinations.

2.3. Synthetic amylose

Synthetic amylose was synthesized with slight modifications as described earlier [15]. In this study synthetic amylose with weight-average molecular mass (M_w) $1.1 \cdot 10^6$ was used Samples were dissolved in 1 *M* NaOH to construct calibration curve for concentration determinations and to establish possible differences in the spectra of amylose–iodine

complex of totally linear synthetic amylose and naturally branched natural amylose.

2.4. Instrumentation

The size-exclusion chromatography (SEC) instrument consisted of a M-590 pump, an M-717 automatic injector, μHydrogel 2000, 500 and 250 (300× 7.8 mm) columns at 70°C. The detector was an M991 diode array detector operating at a wavelength range of 530–795 nm. Post-column iodine addition was done either by pressure-driven Reagent Delivery Module or by an M616 pump.

The eluent was 50 mM NaOH at a flow-rate of 0.5 ml/min and the KI-iodine solution was made by dissolving 0.14 g I_2 and 0.34 g KI in 1 l water buffered with the addition of 0.5% H_3PO_4. The flow-rate for the iodine-solution was 0.3 ml/min.

To determine the molecular mass dual-angle laser light scattering detector (PDI 2000, Precision Detectors, Amherst, MA, USA) and M411 refractive index detectors were employed. Normally 100 μl injections were used but in the case of natural amyloses concentration calibration curves were constructed by injection of various volumes of samples to save material. All instrumentation and columns with the exception of the laser-light scattering detector were from Waters (Milford, MA, USA). The system was controlled and data was handled by Milleniun program from the same source.

2.5. Peak deconvolusion

The 3-chromatogram of convoluted amylose and amylopectin was deconvoluted to individual components by using least-square fit program of Matlab (Matworks, MA, USA) with proprietary algorithm. The deconvolusion was based on the spectra of synthetic amylose (M_w~$1.1\cdot10^6$) and amylopectin sample (M_w~$2\cdot10^6$). The amylopectin was obtained by enzymatically hydrolysing waxy maize starch and by fractionation by column chromatography as described earlier [16].

3. Results and discussions

The basis for the method is shown in Fig. 1 were

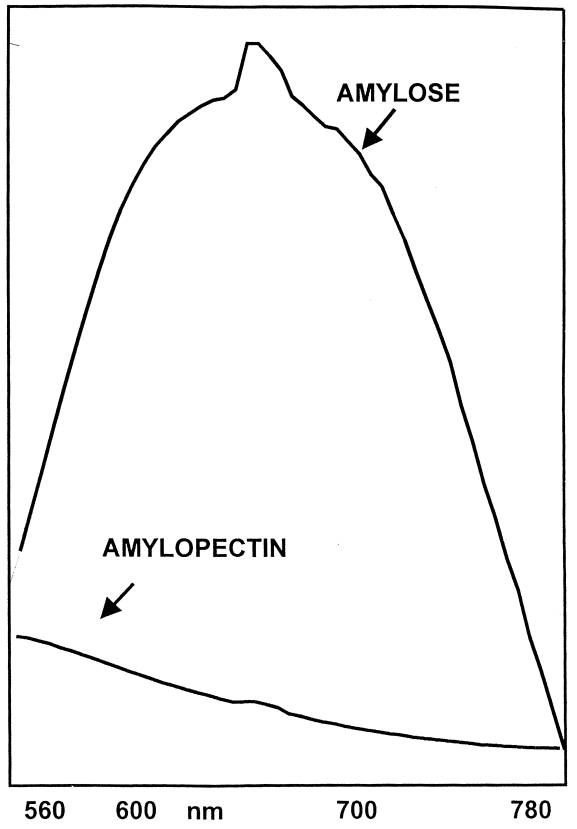

Fig. 1. Spectra of amylose and amylopectin after post-column iodine-colourization. The spectra are recorded at peak apex and correlated to the same scale on the basis of their concentration at peak apex determined by refractive index detector.

spectra of synthetic amylose and waxy maize amylopectin fraction are compared. The absorbance of amylose at 750 nm is about 40× higher on mass basis. Therefore it offers good selectivity for detection, which is essential as the contaminating amylopectin will have higher molecular mass and elute in front of the amylose peak and with an overlapping of the amylopectin and amylose peaks. This could seriously affect the reliability of M_w determination by SEC.

Alkaline compatible columns were used as five years continuous usage has shown them to be very durable. The alkaline eluent keeps columns clean and also after repeated injection of natural samples which are dissolved in 1 *M* NaOH no further manipulations than gentle centrifugation are needed. Therefore they often contain proteins and an overwhelming majority

of small molecules. As the columns are able to withstand to repeated injections of samples dissolved in 1 *M* NaOH and in this high pH, the majority of –OH groups in glucose are ionized so the dissolution of samples is efficient and agglomeration and retrogradation are hindered. Also, there is no effect by lipids and additional sample preparation steps are not required.

The SEC calibration curve (Fig. 2) constructed on the basis of isolated natural amylose fractions was linear ($r^2>0.99$) from 500 000 to 50 000, but laser-light scattering revealed that the linearity can be extrapolated to 2 000 000, which is consistent with results obtained by Roger et al. [7].

Calibration curves (Fig. 3) constructed by the injection of different volumes (100–2 μl) of isolated

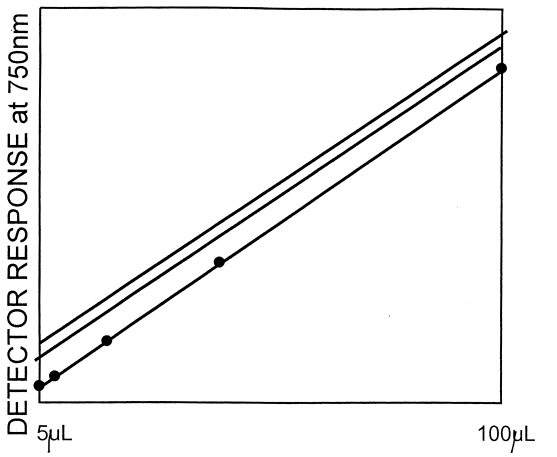

Fig. 3. Concentration calibration curves for amylose fractions having M_w values $5\cdot10^5$, $2.5\cdot10^5$ and $1\cdot10^5$. For further details see text.

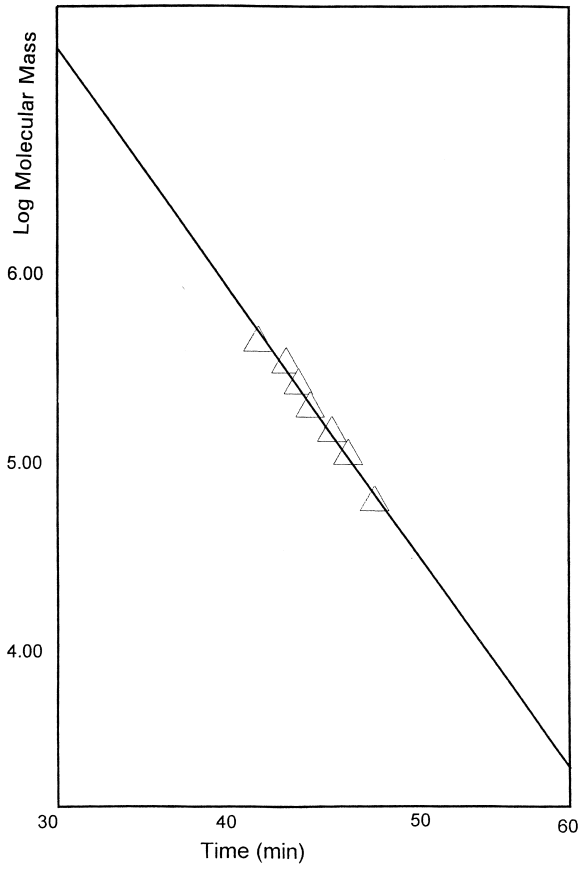

Fig. 2. SEC calibration curve constructed on the basis of isolated barley amylose fraction. For further details see text.

fractions of natural amylose were linear ($r^2>0.999$) at 750 nm as well as at 630 nm and at 560 nm in the concentration range of 2000–8 mg/l. The same was true for 100 μl injections of synthetic amylose ($M_w=1.1\cdot10^6$) in the concentration range 800–4 mg/l. The UV spectra were constant for fractions of natural amylose from M_w 500 000 to 100 000 but a small shift towards lower wavelengths in the $M_w=50\,000$ fraction was observed. This is also reflected in the ratio of (UV-absorbance response)/(refractive index detector response), which remained the same in the fraction from 500 000 to 100 000 but decreased little in the fraction of 50 000 at 750 nm. The synthetic amylose and natural barley amylose had identical spectra (Fig. 4), thus the effect of slight branching occurring in natural amylose has only a negligible effect on spectra. The synthetic amylose having $M_w=26\,000$ show decreased absorbance at 750 nm and a somewhat lower UV maximum than the synthetic amyloses of higher molecular mass.

Increasing and decreasing iodide/iodate concentration by 25% was used to test the systems ruggedness and this had no ill-effect on either peak height or area. Increasing the flow-rate of post-column colourization solution to 0.4 ml/min or decreasing it to 0.2 ml/min had only the effect that can be related to the different dilution of the sample.

In spite of its long working life, the standard

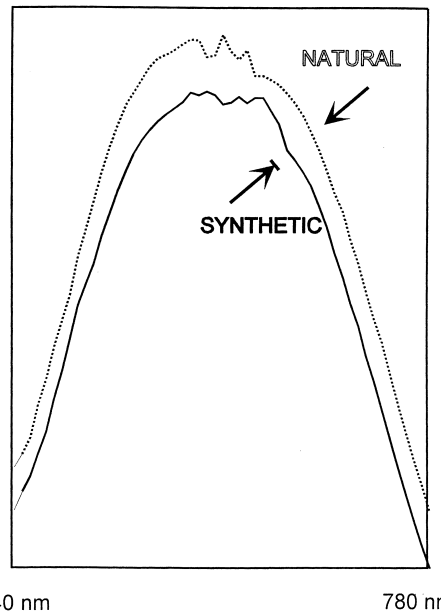

Fig. 4. Spectra of natural barley amylose fraction ($M_w = 5 \cdot 10^5$) and synthetic amylose ($M_w = 5 \cdot 10^5$) after post-column iodine colurization. For further details see text.

deviation of the flow-rate of the M-590 pump, has been measured as less than 0.2% which will be reflected as 3% error in M_w determination.

Table 1 shows the results of quantitative determination of amylose in different starch samples. Taking into account the natural variations in amylose content caused by cultivation conditions, different varieties and also by the different analytical techniques used (especially for sample preparation) the results are in good agreement with results reported in the literature [1] with the exception of potato amylose which gave a much lower value than the other starches. This may be caused by the difficulty of dissolving potato starch in NaOH as compared with cereal starches. The defatting of samples seems to yield 10–20% lower results than direct dissolution in 1 *M* NaOH. This may caused by the increased moisture content of samples after defatting as samples were stored under an ordinary laboratory atmosphere like other starch samples. The other probable explanation is also the incomplete removal of DMSO by washing steps after precipitation.

The results for M_w and number-average molecular mass M_n are also in Table 1. These values are higher than most values reported in the literature. The reason is quite evident as ordinary M_w determination by laser-light scattering requires highly pure sample samples which are normally prepared by aqueous leaching at a temperature only somewhat higher than the gelation temperature in order to avoid sample contamination by amylopectin. As can been seen in Fig. 5 the aqueous leaching of barley starch resulted in noticeably lower molecular mass, as the higher-molecular-mass population of amylose was not leached.

Similar results are also reported also by Aberle et

Table 1
Results from determination of amylose content and weight-average (M_w) and number-average (M_n) molecular masses of various starches

	Treatment	Amylose (%)	$M_w \cdot 10^3$	$M_n \cdot 10^3$
Wheat		32	1700	300
Wheat	Defatted	23	2100	400
Rye		32	1300	300
Rye	Defatted	26	1500	400
Barley		32	700	200
Barley	Defatted	27	800	200
Barley starch	Leaching at 95°C		500	200
Amylomaize		59	400	100
Amylomaize	Defatted	55	500	100
Oat		32	1700	400
Oat	Defatted	26	2000	400
Oat	Leaching at 95°C		500	200
Maize		30	1200	200
Maize	Defatted	27	1200	200
Potato		10	3000	500

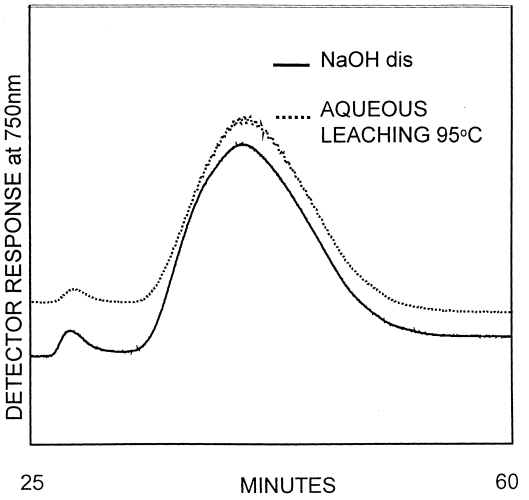

Fig. 5. Chromatogram of barley starch dissolved by aqueous leaching at 95°C or by direct dissolution in 1 *M* NaOH. Detection at 750 nm. For further details see text.

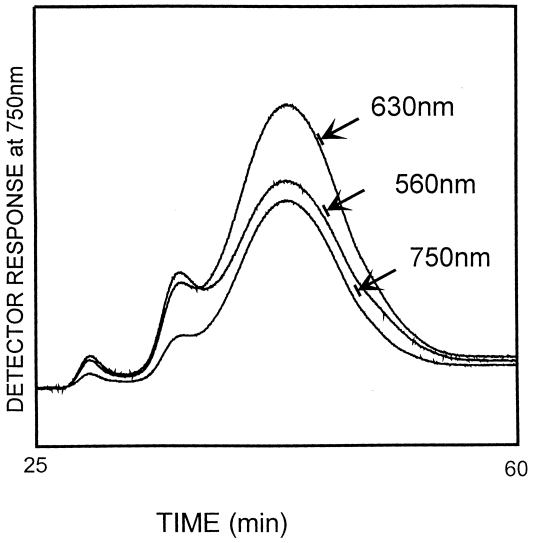

Fig. 6. Chromatogram of oat starch monitored at 750 nm, 630 nm and at 560 nm. For further details see text.

al. [17], by Mua and Jackson [18] and by Klinger and M. Zimbalski [19] by the application of more rigorous conditions for amylose dissolution.

The spectra of amylose in all samples starch remained constant over the entire peak with the exception of potato. The peak of potato amylose showed a slight tendency for lower λ_{max} at higher M_w region, but this can be also partly due to artifacts caused by the absorbance of iodide/iodine solution because of low absorbance of potato sample.

In the chromatogram of barley a contaminating peak could be seen at retention time ~32 min (Fig. 5). Comparing the chromatograms (Fig. 6) at different wavelengths will reveal that this sample is contaminated with a high amount of amylopectin. The Fig. 7 shows the same chromatogram after the peaks for amylose and amylopectin are mathematically deconvolved. In the same figure is also the sum curve of these deconvolved peaks showing a good match with original peak-shape. Thus using this procedure, amylose peaks which are so highly contaminated by amylopectin that it has a visible effect on peak shape, may also be analysed.

It has been shown by Roger et al. [7] that the branching has only a negligible effect on elution of amyloses so the SEC calibration curve may be constructed on the basis of fractions of natural amylose, whose M_w can be reliably determined, as

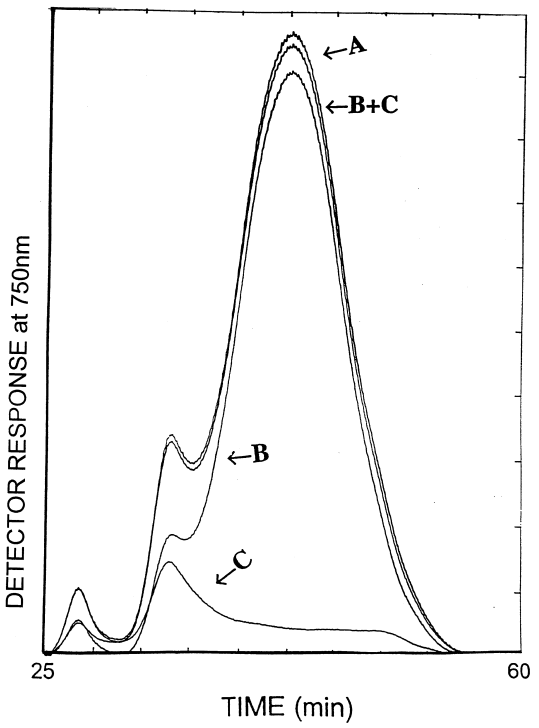

Fig. 7. Same chromatogram as in Fig. 6 but after the peaks of amylase and amylopectin are mathematically deconvolved.

synthetic amyloses of different M_w are not commercially available. As the limit between linear amylose, branched amylose and so-called intermediate material is vague the use of synthetic amylose or highly purified natural amylose (with high enough M_w) at specific wavelengths for the measurement of amylose content of starch has obvious advantages. It targets the measurement on those molecules containing long linear chains and which have most effect on certain qualities of amylose–amylopectin mixtures. The method described here also allows very simple and straightforward sample preparation where the complications arising from agglomeration, retrogradation and complexes are avoided. The development of chromatographic workstations will in the future offer more easily accessible refined tools like mathematical peak and spectral deconvolusion with which starch samples which contain very high amounts of low-molecular-mass amylopectin, that cannot be separated chromatographically, can also be analyzed.

References

[1] S. Hizukuri, in: A.-C., Eliasson (Ed.), Carbohydrates in Food, Marcel Dekker, New York, Basel, 1993, ch. 9, p. 375.
[2] M.J. Gidley, P.V. Bulpin, Macromolecules 22 (1989) 341.
[3] P. Roger, P. Colonna, Carbohydr. Polym. 21 (1993) 83.
[4] S. Lansky, M. Kool, T.J. Schoch, J. Am. Chem. Soc. 71 (1949) 4066.
[5] N. Hawort, S. Peat, P.E. Sargott, Nature 1 (1946) 19.
[6] Y. Takeda, S. Hizukuri, B.O. Juliano, Carbohydr. Res. 148 (1986) 299.
[7] P. Roger, V. Tran, J. Lesec, P. Colonna, J. Cereal Chem. 24 (1996) 247.
[8] N.K. Matheson, L.A. Welsh, Carbohydr. Res 180 (1988) 50.
[9] E.J. Bourne, W.N. Haworth, A. Macey, S. Peat, J. Chem. Soc. (1948) 924.
[10] W. Banks, C.T. greenwood, D.D. Muir, Stärke 23 (1971) 118.
[11] B.L. Larsson, K.A. Gilles, R. Jennes, Anal. Chem. 25 (1953) 802.
[12] T. Suortti, O. Pessa, J. Chromatogr. 536 (1991) 251.
[13] W. Thorn, GIT Fachz. Lab. 10 (1990) 1255.
[14] P. Roger, P. Colonna, Carbohydr. Polym. 21 (1993) 83.
[15] P. Roger, P. Colonna, Carbohydr. Res. 227 (1992) 73.
[16] P. Parovuori, R. Manelius, T. Suortti, E. Berthoft, K. Autio, Food Hydrocolloids 11 (1997) 471.
[17] Th. Aberle, W. Burchard, W. Vorwerg, S. Radosta, Starch/Stärke 46 (1994) 329.
[18] J.P. Mua, D.S. Jackson, Cereal Chem. 72 (1995) 508.
[19] R.W. Klingler, M. Zimbalski, Starch/Stärke, (1992) 44.