Journal of Thermal Analysis and Calorimetry, Vol. 86 (2006) 2, 291–301

STRUCTURE OF STARCHES EXTRACTED FROM NEAR ISOGENIC WHEAT LINES
Part I. Effect of different GBSS I combinations

S. S. Kozlov[1], *T. Noda*[2], *E. Bertoft*[3] *and V. P. Yuryev*[1*]

[1]Institute of Biochemical Physics, Russian Academy of Sciences, Kosygina st. 4, 119991 Moscow, Russia
[2]Department of Upland Agriculture, National Agricultural Research Center for Hokkaido Region, NARO, Shinsei, Memuro Kasai, Hokkaido 082-0071, Japan
[3]Department of Biochemistry and Pharmacy, Abo Akademi University, BioCity, P.O. Box 66, 20521 Turku, Finland

Differential scanning calorimetry (DSC), acidic hydrolysis and different physico-chemical approaches were used to study thermodynamic and structural characteristics of starches from near-isogenic wheat lines to establish the effect of different combinations of active granule-bound starch synthase isoforms, taking part in amylose biosynthesis, on the structure and thermodynamic properties of starches. Obtained results suggest that the effect of different GBSS I combinations is realized through altered amylose localization within starch granules, reflecting in changes of melting temperature of crystalline lamellae (T_m) and rates of acidic hydrolysis. It has also been demonstrated that changes in T_m values for native wheat starches are determined by amylose content in amylopectin clusters.

Keywords: *acidic hydrolysis, amylose localization, DSC, granule-bound starch synthase, wheat starches*

Introduction

In spite of latter achievements in understanding of enzymatic reactions occurring during biosynthesis of starch polysaccharides (amylose, amylopectin), the processes of formation of starch semi-crystalline structures are poorly known. According to classical approaches of physical chemistry, the structure of polymer crystals depends on the degree of polymerization of molecules, their concentration in mother water as well as on temperature conditions of crystallization. These presentations have found acknowledgement at investigation of starch spherulitic crystals [1, 2] and the structure of starches grown at different environmental conditions [3, 4]. Particularly, it has been shown that rise of environmental temperature leads to formation of more perfect starch crystals with higher melting temperatures. If perfection of starch crystals is determined only by conditions of crystallization, annealing of starches extracted from one cultivar but grown at different environmental temperatures should eliminate difference in melting temperatures. However, the analysis of published data shows that this difference remains sufficiently significant for annealed starches [5, 6]. As starch is biological object, it could be supposed that besides physico-chemical factors genetic factors can also exert influence on structure of crystals forming during starch biosynthesis. Generally, it can be proposed that there is a relationship between the number of active enzymes, controlling amylose and amylopectin biosynthesis, their activity and starch granule structure. The hypothesis concerning influence of activity of different isoenzymes on structure of starches has been also noted by Tester [7]. At present, however, these hypotheses are lacking in experimental evidences.

Models of different levels of starch granule organization and assembly structures have been proposed [8–16]. At present, the cluster model giving adequate description of the structure of amylopectin and normal starches is generally accepted. According to the model, the structural periodicity in semi-crystalline starch granules is formed by repeating layers of amorphous background, consisting of amylopectin and amylose macromolecules in unordered conformation, and semi-crystalline growth rings. The latter consist of periodically arranged crystalline and amorphous lamellae formed by amylopectin clusters. For amylopectin and normal starches the overall thickness of the cluster is 9–10 nm [8, 12, 14, 16] and the thickness of crystalline lamella is ~5–6 nm [16–18]. The crystalline lamella consists of crystallites formed by ordered double helices from amylopectin A-chains [8–18]. In contrast to the crystalline lamella, the amorphous lamella consists of long amylopectin B-chains [15, 17]. The detailed analysis of the model shows that the role of amylopectin macromolecules in the structural organization of starch granules is comparatively well known, whereas the function of the amylose and its exact localization in starch granules remains uncertain [19–21].

* Author for correspondence: v.yuryev@sky.chph.ras.ru

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Recently it has been shown that Naegeli dextrins (amylopectin clusters) extracted after the first stage of acidic hydrolysis of different starches contain amylose molecules [22]. These results have confirmed the assumption published earlier [17, 18, 23, 24] that amylose molecules play the role of defects located within amylopectin clusters and penetrating both crystalline and amorphous lamellae. In amorphous lamellae these amylose molecules are in unordered conformation, while in crystalline lamella the same molecules are reasonably rigid ('string-type') [23]. Such molecules are generally called amylose 'tie-chains' and could be considered as defects in respect to the amylopectin clusters.

Biosynthesis of native starch granules under a decrease of soil temperature during maturation of starch containing plants leads to accumulation of defects (amylose 'tie-chains') in starch granules and a decrease of the melting temperature of starch crystalline lamellae [3, 18]. Additionally, when passing from amylopectin to normal and further to amylose rich (30–50% amylose) wheat starches, decrease in the melting temperature of crystalline lamellae is observed [25]. The latter result is expected since according to the fusion theory of semi-crystalline synthetic polymers, melting of crystals begins from their defects [26, 27]. However, such behavior is not observed for all starches. Particularly, for wheat starches from near isogenic lines an increase of amylose content from 2.1 to 19.5% is accompanied by insignificant changes in the melting temperature of crystalline lamellae, while further increase of amylose leads to drastic decrease of the melting temperature [28]. Considering the fusion theory of semi-crystalline polymers and the cluster model for structure of starch granules [8, 12, 14–16, 26], it could be supposed that these differences are due to different localization of amylose molecules in amylopectin cluster. However, calorimetric data in the work of Mangalika *et al.* [28] were obtained at quite high concentrations of starch-water dispersions (30%). It means that the melting process of starch crystalline lamellae could not be considered as quasi-equilibrium (the influence of concentration of starch dispersions on melting properties of starches is well known [17]), and it does not allow to use different physical approaches for description of the melting process of starches. Since the environmental factors during maturation were the same for all wheat cultivars described [28], it could be supposed that hypothesized altered localization of amylose macromolecules is due to differences in enzymatic activity controlling amylose biosynthesis, namely, due to different combinations of active granule-bound starch synthase (GBSS I) isoforms.

GBSS I, also known as 'waxy' protein, is the key enzyme for amylose biosynthesis [29]. In bread wheat, three GBSS I isoforms (Wx-A, Wx-B, Wx-D proteins) are encoded by three waxy loci, Wx-A1, Wx-D1, and Wx-B1 [30]. Presence of one or more non-functional GBSS I alleles (null alleles) leads to loss of one or more GBSS I isoforms resulting in reduction or absence of amylose in starch granules. By purification and electrophoretic separation of wheat GBSS I, the identification of the protein products of the three Wx loci was possible [31], showing the existence of GBSS I polymorphism among wheat accessions and allowing the identification of waxy wheat types. The efficiency of each locus on amylose production is different, the highest potency shown by Wx-B1, followed by Wx-D1 and Wx-A1 [32, 33]. At present, intensive investigations concerning GBSS I, structure, thermodynamic and functional properties of wheat starches are realized worldwide [25, 28–33]. Irrespective of differences in proposed mechanisms of amylose biosynthesis [34, 35], it is supposed that GBSS I located in amylopectin matrix elongate amylose chains that are pulled out to amorphous lamellae and/or amorphous background. However, the problems concerning influence of different combinations of active GBSS I isoforms on structure and thermodynamic properties of starches remain uncertain. Solution of these problems is important since it provides valuable information about the role of GBSS I enzymatic activity in structure formation of starch granules [36].

The aim of this work is experimental confirmation of the hypothesis concerning the effect of different combinations of GBSS I isoenzymes on the thermodynamical and structural properties of wheat starches from near-isogenic lines (containing one, two or three null GBSS I alleles, i.e. single, double and triple null wheat lines). Different physico-chemical approaches usually applied for studying structural features of synthetic polymers and starches, as well as acidic hydrolysis of starches, were used to clarify the proposed hypothesis.

Experimental

Materials and methods

Plant materials

A spring wheat cultivar Chinese Spring (CS) and its near-isogenic partial and waxy lines developed through a doubled-haploid method were used [37]. The Wx protein profiles of wheat types were studied elsewhere [28] and are shown in Table 1. Each of the seven types was grown at the same environmental conditions at the National Agricultural Research Center for the Hokkaido region, Hokkaido, Japan in 2001 under standard field management. The plants were

Table 1 Wx protein profile of wheat types from near-isogenic lines, amylose content in extracted starches, cooperative melting unit (ν) and thickness of crystalline lamellae (L_{crl}) for investigated wheat starches

Wheat type	Wx protein profile*			Amylose content/%	ν/anhydroglucose residue	L_{crl}/nm
	Wx-A1	Wx-B1	Wx-D1			
Wx-A	–	+	+	22.4	14.7	5.1
Wx-B	+	–	+	22.0	15.5	5.4
Wx-D	+	+	–	21.1	13.2	4.6
Wx-AB	–	–	+	18.6	16.7	5.8
Wx-AD	–	+	–	19.5	13.4	4.7
Wx-BD	+	–	–	17.1	15.8	5.5
Wx-ABD	–	–	–	2.1	12.3	4.3
Mean value					14.6±1.5	5.1±0.6

*Wx protein profiles were determined in the work [28]. (+) and (–) indicates presence and absence of Wx proteins

covered by a transparent plastic roof to prevent rain damage two weeks before maturity. Plants were individually harvested at maturity. Usually in work of such type, starches from wild type wheat are considered as control object for investigation. However, since amylose synthesis in wild type wheat is controlled by three GBSS I isoenzymes, it will undoubtedly only put additional difficulties due to complication of system investigated. Therefore we exclude advisedly starch extracted from wild type wheat at the first stage of the investigation.

Starch preparation

Grain samples of each waxy type were milled on experimental mill (Buhler Inc., Uzwil, Switzerland) to produce 60% extraction. Flour (50 g) was mixed with ~30 mL of water to form a dough ball, which was kept in cold distilled water for 1 h. The dough ball was then kneaded in water until all starch granules were extracted. The starch suspension was sieved using a 45 mm sieve. The filtrate was allowed to stand for at least 3 h to precipitate starch. The supernatant was discarded and the starch sediment was washed twice with water and 80% acetone, and then air-dried. The moisture content (~12% for all starches) was estimated by oven-drying of 1 g of sample at 115°C for 3 h.

Amylose content

The amylose content in native starches was determined by the Concanavalin A method [38] using an amylose/amylopectin assay kit (Megazyme Inc., Wicklow, Ireland). The analysis was repeated five times. The amylose content in native starches is shown in Table 1.

Apparent amylose content in crystallites produced after acidic hydrolysis of native starches was determined by iodine-binding as described in Shibanuma *et al.* [39]. The blue value at 680 nm of absorption spectra of iodine starch complex was determined by using Beckman DU-640 spectrophotometer. As Shibanuma *et al.* reported, the average BVs of amylose and amylopectin isolated from five wheat samples were 1.24 and 0.100, respectively, these BVs were used in the calculation of the apparent amylose content [39].

Lintnerization

Starch granules were solubilized with 2.2 M HCl (0.025 g mL^{-1}), according to Robin *et al.* [8], at 29.5°C. During lintnerization, the samples were shaken periodically. The samples were taken at determined time intervals (24 h) whereupon the supernatant was discarded and the sediment was washed out by water to pH 6.5. The amount of starch was estimated by drying the sediment at 115°C to constant mass. The amount of solubilized substance formed during lintnerization of native starches (S/%) was calculated from Eq. (1) as:

$$S = \frac{A_0 - A_{dw}}{A_0} \cdot 100\% \quad (1)$$

where A_0 is the initial mass of starch and A_{dw} is the quantity of unsolubilized starch (dry mass). After 13 (for Wx-B starch) and 15 (for Wx-AB and Wx-ABD starch) days of hydrolysis the samples were taken, washed out by water to pH 6.5 and then dried at room temperature.

High-sensitivity DSC

Calorimetric investigations of starch dispersions in water (0.3% dry matter, sample volume 0.5 cm^3 in sealed cells) were performed using a high sensitivity differential scanning microcalorimeter DASM-4 (Puschino, Russia) over the temperature range of 10–120°C with a heating rate of 2 K min^{-1} and excess pressure of 2.5 bar. Deionized water was used as a reference material. The heat capacity scale was calibrated using the Joule–Lenz effect for each run. It was shown previously, that under experimental conditions used, correc-

tions for dynamic lag and residence of the samples in calorimetric cell were not necessary, moreover, gelatinization of starch-water dispersions could be considered as quasi-equilibrium process [40]. Additionally, for starches with symmetric DSC curves, the two-state model is applicable for the description of the melting process of crystalline lamellae [40, 41]. This model implies that there is a reversible transition between native and molten states. Accordingly, parameter of cooperativity, which corresponds to the minimal number of monomers undergoing the transition, could be determined [23, 40, 41].

The melting temperature (T_m) was attributed to the temperature of the maximum on the DSC curve. The heat capacity jump (ΔC_p^{exp}) during the melting process was determined by linearly extrapolating the partial heat capacity change of the native C_p^n and molten C_p^m states to the melting temperature T_m and was calculated as follows:

$$\Delta C_p^{exp} = C_p^m - C_p^n \quad (2)$$

Calorimetric enthalpy (ΔH_m) was determined as the area under the peak above the extrapolation lines. The average values of the thermodynamic parameters were determined using five measurements at 95% significance level and normalized per mole of anhydroglucose units (162 g mol^{-1}). The error in determination of T_m is 0.1 K, values for ΔH_m and ΔC_p^{exp} were determined with the error of not more than 5%.

Values for van't Hoff enthalpy (ΔH^{vH}) were calculated according to previously published papers [17, 23, 41] as:

$$\Delta H^{vH}=2R^{1/2}T_m(C_p-0.5\Delta C_p^{exp})^{1/2} \quad (3)$$

where R is gas constant, T_m is the melting temperature of starch crystalline lamellae, C_p is the difference between the maximum ordinate on the DSC curve and the value of C_p^n, linearly extrapolated to the melting temperature T_m. Values for the parameter of cooperativity (ν) and the thickness of crystalline lamellae (L_{crl}) were calculated according to the following equations:

$$\nu=(\Delta H^{vH})/(\Delta H_m) \quad (4)$$

$$L_{crl}=0.35\nu \quad (5)$$

where ΔH_m is the experimental melting enthalpy of crystalline lamellae and ΔH^{vH} is the van't Hoff enthalpy; the pitch height per anhydroglucose residue in the double helix is 0.35 nm [42].

Results and discussion

Original DSC curves related to the melting of aqueous wheat starch dispersions (Fig. 1) show the typical endothermic transitions [17, 18, 24, 25, 40, 43]. The low-temperature endothermic transition is attributed to melting of the crystalline lamellae, while the high temperature peak is ascribed to the dissociation of the amylose–lipid complexes and/or the melting of single-helical V_h-type crystallites. Because of low amylose content in amylopectin starch from waxy wheat, the second transition is absent for this type of starch. Generally, all the thermodynamic melting parameters related to both crystalline lamellae and amylose–lipid complexes (Figs 1 and 2) are in agreement with previously published data for wheat starches [14, 17, 23, 25, 28, 43–45].

The decreasing trend both in the melting enthalpy and the melting temperature of crystalline lamellae is observed with increasing amylose content in native wheat starches (Fig. 2). According to previously published works [25, 28, 46], decrease in the melting enthalpy can be explained by decreasing degree of crystallinity in starches usually observed when passing from amylopectin to normal and further to amylose-rich wheat starches. In contrast to changes observed in the melting enthalpy, an explanation for a decrease of the melting temperature is a comparatively complex problem. These changes can be due to three different but, apparently, interconnected reasons namely: (*i*) genetic factors, i.e. different activities of starch-synthesizing enzymes, (*ii*) differences in growth temperature during maturation of starch containing plants [3, 47], and (*iii*) differences in localization of amylose macromolecules inside starch granules.

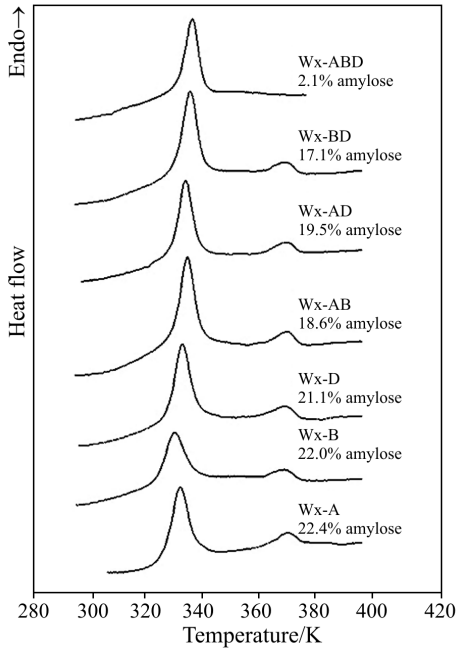

Fig. 1 DSC curves of water dispersions of wheat starches with different amylose content

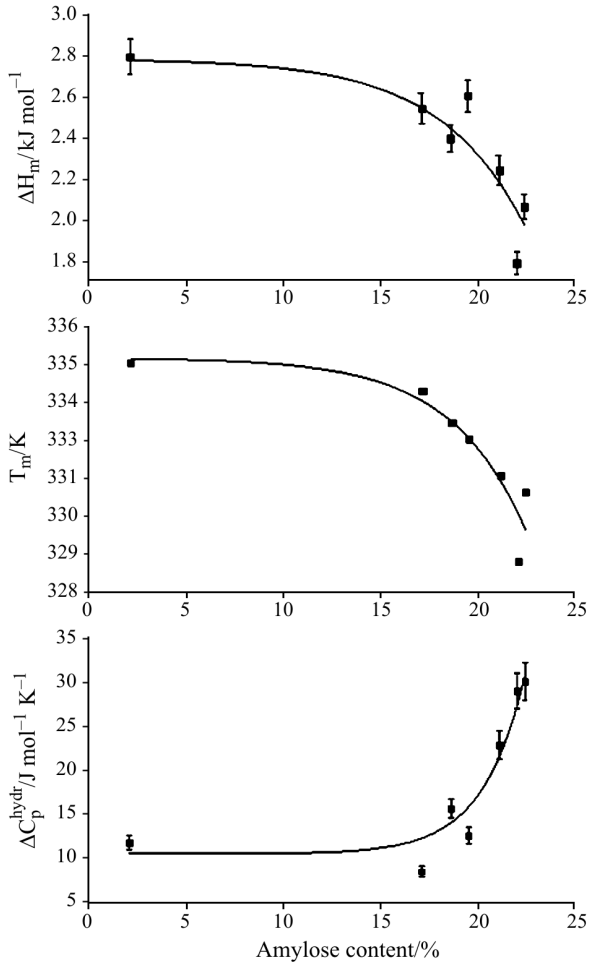

Fig. 2 Thermodynamic melting parameters of crystalline lamellae *vs.* amylose content in native mutant wheat starches (ΔC_p^{hydr} – heat capacity jump related to starch hydration during gelatinization, Eq. (7))

The first reason could be rejected as the starches were from near isogenic lines and have common genetic background. It is well known that smallest potency in amylose production is shown by Wx-A1a allele, followed by Wx-D1a and Wx-B1a alleles [33, 37]. The conclusion is in agreement with the results presented in Table 1 for the investigated starches. Comparison of data presented in Table 1 for wheat types containing Wx-A1a, Wx-D1a and Wx-B1a alleles (i.e. double null lines) with their melting temperatures shows that a decrease of the melting temperature of starches are in agreement with a potency of synthases producing amylose during biosynthesis. This could mean that the thermodynamic melting parameters of crystalline lamellae principally depend on the type of amylose-producing synthases. A decrease in the melting temperature of starches can also be due to an accumulation of defects in crystalline lamellae because of decrease in soil temperature during the growth of starch containing plants [17, 18]. However, it is worth noticing that the investigated wheat cultivars were grown at the same environmental and soil conditions, so the second reason also does not take place.

According to the theory for semi-crystalline synthetic polymers [26], the melting temperature can be calculated using the Thomson–Gibbs' equation:

$$T_m = T_m^0[1 - 2\gamma_i/(\Delta H_m^0 \rho_{crl} L_{crl})] \qquad (6)$$

where T_m^0 and ΔH_m^0 are the melting temperature and the melting enthalpy, respectively, of a hypothetical crystal with unlimited size (a perfect crystal), γ_i is the free surface energy of face side of crystalline lamellae, while ρ_{crl} and L_{crl} are the density and the thickness of the crystal, respectively. The analysis of Eq. (6) shows that the melting temperature of semi-crystalline synthetic polymers is a function of three following variables: the polymorphous structure of starches, the thickness of crystalline lamellae (L_{crl}) and the free surface energy of crystals face side (γ_i). The γ_i value is mainly governed by the surface entropy that is proportional to the content of defects [26]. Since starch granules are related to semi-crystalline compounds, the application of this equation to describe the changes in melting temperature of crystalline lamellae is acceptable. In fact, this approach was already applied for a description of thermodynamic properties of a number of starches [17, 18, 25]. It is well known that polymorphous structure of wheat starches remains invariable in the range of amylose content from 1.5 to 39.5% [25, 29, 46]. Moreover, the calculations of the cooperative melting unit and the thickness of crystalline lamellae for the investigated starches show that, irrespective of amylose content, values for cooperative melting unit (ν) and L_{crl} values are constant (ν=14.6±1.5 anhydroglucose residues, L_{crl}=5.1±0.6 nm; Table 1) and does not depend significantly on the combinations of active GBSS I isoforms. The conclusion is in agreement with data for other cultivars of wheat starches (ν=13.3±1.6 anhydroglucose residues, L_{crl}=4.7±0.5 nm) [17, 23, 25]. It follows that changes in the melting temperature of crystalline lamellae on increase of amylose content for investigated starches is due to changes in the values of the free surface energy of crystals face side (γ_i).

The fact that defects exist within wheat starches could be confirmed through the estimation of the hydration contribution to the overall heat capacity jump ΔC_p^{exp}. According to Matveev *et al.* [23], experimental heat capacity jump (ΔC_p^{exp}) observed during gelatinization of native starches is the sum of two components, the first related to glass-transition of amorphous polysaccharide chains (ΔC_p^{gtr}) and the second attributed to hydration of polar groups in starch macromolecules during melting of crystalline lamellae (ΔC_p^{hydr}). It is known, that glass transition temperature

(T_g) for starch polysaccharide chains located in amorphous background at excess water is in the range from –45 to –20°C [17, 48]. Additionally, glass-transition process of unordered polysaccharide chains within amorphous lamellae is in same temperature interval as melting process of crystalline lamellae, with the T_g to T_m ratio being in the range from 0.5 to 0.97–1 [17, 23, 24, 49]. It was shown previously [17, 23], that the ΔC_p^{gtr} value is 12.5±2.8 J mol^{-1} K^{-1}, irrespective of starch origin and polymorphous structure, being in accordance with the law of constant heat capacity jump proposed by Wunderlich for low and high molecular mass organic glasses [50]. Hence, ΔC_p^{hydr} could be evaluated as follows:

$$\Delta C_p^{hydr} = \Delta C_p^{exp} - \Delta C_p^{gtr} \quad (7)$$

where ΔC_p^{exp} and ΔC_p^{gtr} are the experimentally determined heat capacity jump across the melting curve of native starches and the heat capacity jump related to the glass-to-rubber transition, respectively. As can be seen from Fig. 2, the changes in the ΔC_p^{hydr} *vs.* amylose content can be approximated by exponential function with reasonably high correlation coefficient (R=0.923). As was previously shown, ΔC_p^{hydr} for most normal starches, including wheat starches selected through usual breeding, does not exceed 18.5 J mol^{-1} K^{-1} [17, 23, 25]. As can be seen from Fig. 2, similar values are obtained for starches containing from 1.5 to 19.5% while further increase of amylose content leads to an increase of the ΔC_p^{hydr} values. For waxy and normal maize, and for high amylose barley starches, ΔC_p^{hydr} is in the 27.1 to 56.1 J mol^{-1} K^{-1} range. Such differences, according to Yuryev *et al.* [17] and Matveev *et al.* [23], can be related to the higher content of defects (amylose tie-chains, in particular) in crystalline lamellae of maize and barley starches. As can be seen from Fig. 2, the ΔC_p^{hydr} values for the investigated starches increase with rise of amylose content in starches and reach maximal values of approximately 29 J mol^{-1} K^{-1}. These values are comparable with those for maize and barley starches that have high amount of defects. Summarizing obtained data, it could be stated that at an increase of amylose content in wheat starches, an increase of the ΔC_p^{hydr} values is due to an accumulation of defects such as amylose 'tie-chains'. Taking into consideration the mechanisms of amylose biosynthesis proposed by Smith *et al.* [34] and Ball *et al.* [35], amylose 'tie-chains' correspond to amylose chains 'frozen' in crystalline lamellae, i.e., amylose chains that are not pulled out to amorphous lamellae and/or amorphous background during structure formation of starch granules. As can be seen from Fig. 2, the most significant accumulation of defects in starches is apparently observed at amylose content of >19.5%. Additionally, our calculations show that changes in the melting temperature of crystalline lamellae correlate satisfactorily (correlation coefficient of linear regression is –0.89) with changes in the ΔC_p^{hydr} for the starches investigated. This means that these parameters (T_m, ΔC_p^{hydr}) can be considered as interconnected.

Taking into consideration: (*i*) Eq. (6) describing the changes in melting temperature of semi-crystalline synthetic polymers and some starches; (*ii*) that at increased amylose content ΔC_p^{hydr} correlates with the content of defects [17, 23]; (*iii*) that there is an interconnection between the T_m and the ΔC_p^{hydr} values, it could be supposed that the trend in changes of the melting temperature of crystalline lamellae (Fig. 2) is determined by an accumulation of amylose 'tie-chains'.

The analysis of changes of thermodynamic parameters (T_m and ΔC_p^{hydr}) shows that depending on amylose content, the investigated starches can be subdivided into three groups. Amylopectin starch can be related to first (control) group, amylose synthesis in amylopectin starch is not controlled by known synthases (triple null line starch). The second ('transitional' group) include starches containing 17.1, 18.6 and 19.5% amylose, i.e. starches with insignificant deviations in thermodynamic parameters in comparison with the data for amylopectin starch. In starches from the second group amylose biosynthesis is controlled by only one GBSS I isoform (double null line starches), encoded by Wx-A1, Wx-B1 or Wx-D1 loci, depending on wheat cultivar. Starches containing >21% amylose are related to the third group. For these starches, there are large deviations of the thermodynamic parameters in comparison with amylopectin starch. Two GBSS I isoenzymes (encoded by Wx-A1 and Wx-B1, Wx-A1 and Wx-D1, or Wx-B1 and Wx-D1 loci) take part in amylose biosynthesis of starch from the third group (single null line starches). Taking into account that: (*i*) the fusion of crystals begins from defects [26, 27, 50], (*ii*) as a rule, defects are associated within amylopectin clusters [17, 18, 23, 24], (*iii*) the ΔC_p^{hydr} values characterize the content of defects in crystalline lamellae [17, 23, 25], (*iv*) in contrast to single null line starches, the T_m and ΔC_p^{hydr} values for triple and double null line starches are similar to each other (Figs 2b and c), it can be supposed that amylose macromolecules in double null line starches are predominantly located in the amorphous background and/or amorphous lamellae, oriented transverse to the lamellar stack, according to Gidley's opinion [51]. Amylose molecules in single null line starches can be located both in amylopectin clusters, playing the role of defects, and in amorphous background/amorphous lamellae. It is worth noting that amylose macromolecules located within amorphous lamellae could not be considered as defects and, respectively, could not influence on the melting temperature of crystalline lamellae [52]. The different localization of

amylose macromolecules was confirmed through the acidic hydrolysis of starches.

Figure 3 shows the typical curves of solubilization process of starch in the diluted hydrochloric acid as a function of time [8, 21, 53–55]. As can be seen from Fig. 3, irrespective of amylose content in starches, the hydrolysis proceeded in two stages that can be approximated by two linear functions. The first is ascribed to hydrolysis of macromolecules located in the amorphous background and possibly in amorphous lamellae, while the second function describes hydrolysis of macromolecules arranged in crystallites. After the first stage of hydrolysis, when amorphous regions of the granules are almost hydrolyzed, crystallites could be extracted (Fig. 3, open symbols) and further analyzed by DSC (Fig. 4). The rates of acidic hydrolysis of starches on first and second stages, content of substances located in amorphous regions and apparent amylose content in crystallites produced after acidic hydrolysis ($AC_{crystall}$) are presented in Table 2. Presence of amylose macromolecules in crystallites is expected. For example, the differences in the amylose content of crystallites produced after acidic hydrolysis of different legume starches were determined in the previous paper [54]. It was shown, in particular, that crystallites from wrinkled pea (64% amylose) and lentil (45% amylose) contained ~36 and ~16% amylose, respectively, while crystallites from adzuki bean and smooth pea lacked amylose macromolecules.

The rates of starch hydrolysis on both stages (1st stage – hydrolysis of amorphous background and amorphous lamellae, 2nd stage – hydrolysis of crystallites) of the process should depend on the localization of amylose macromolecules (amorphous background or amylopectin clusters) as well as on the rate of penetration of H_3O^+ ions into granules through semi-crystalline growth rings. As noted above, single null line starches apparently contain a larger amount of defects. Since these defects play the role of 'channels' facilitating penetration of H_3O^+ ions inside the granule, the rate of acidic hydrolysis on both stages of process should be higher for single null line starches, whereas the rates of hydrolysis for double and triple null line starches should be lower. As can be seen from Table 2, on both stages the rates of acidic hydrolysis for Wx-B starch (single null line starch) is higher (on 23.6% at the first stage and on 29.7% at the second stage) in comparison with the corresponding rates for Wx-ABD (triple null line starch) and Wx-B (double null line starch) starches. Considering the errors in the measurements, the rates of hydrolysis for double and triple null line starches are equal. It follows that at

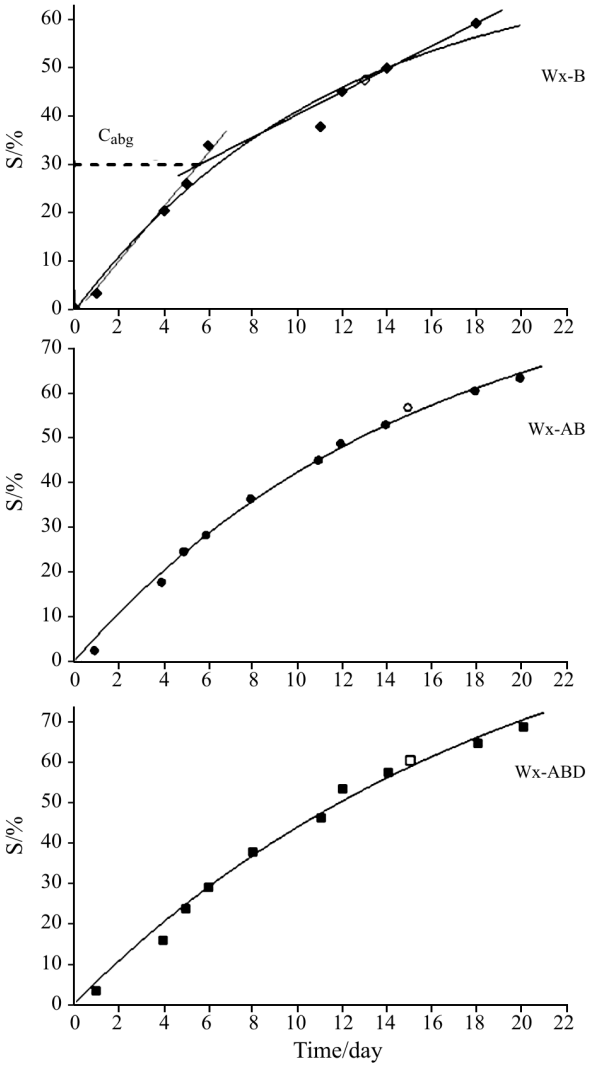

Fig. 3 Lintnerization of wheat starches with different amylose content. Open symbols mark the samples that were taken for further analysis

Table 2 Amylose content in native wheat starches, rates of acidic hydrolysis on first (r_{abgr}) and second ($r_{amylopeccl}$) stages, content of substances located in amorphous regions (C_{abg}), apparent amylose content in crystallites produced after acidic hydrolysis ($AC_{crystall}$)

Wheat type	Amylose content/%	r_{abgr}, amount of dissolved substances/% day^{-1}	$r_{amylopeccl}$, amount of dissolved substances/% day^{-1}	C_{abg}/%	$AC_{crystall}$/%
Wx-B	22.0	5.5±0.3* (0.995**)	2.42±0.01 (0.999)	28±4	5.4
Wx-AB	18.6	4.0±0.3 (0.989)	1.6±0.2 (0.978)	47±7	2.1
Wx-ABD	2.1	4.4±0.2 (0.993)	1.8±0.2 (0.992)	55±5	~0

*errors in determination of the rates of acidic hydrolysis are given for 95% confidence interval, **correlation coefficient of linear regressions, describing experimental data is indicated within parenthesis

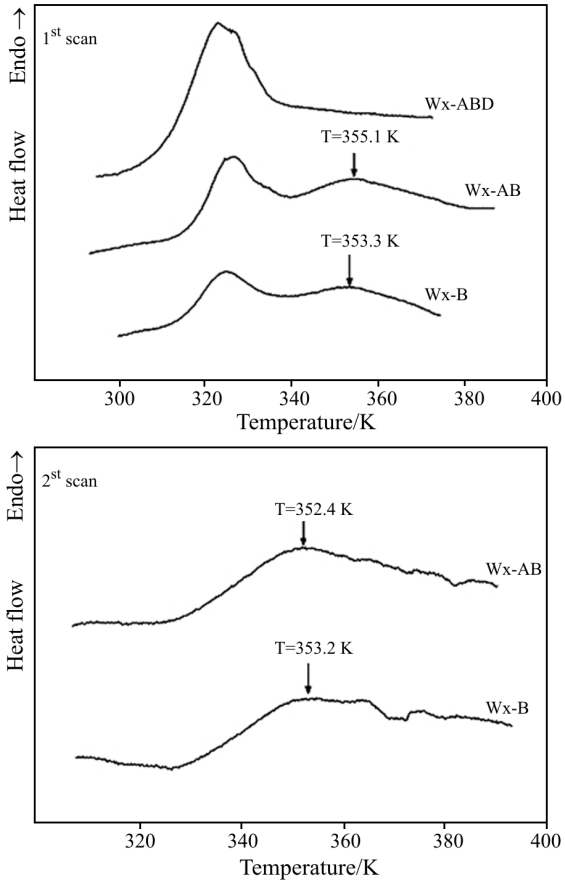

Fig. 4 DSC traces of lintnerized wheat starches. Arrows indicate the endothermic transition attributed to dissociation of amylose–lipid complexes

first approach we can indeed assume that the amylose molecules in the latter case are predominantly located in amorphous regions, while for single null line starches amylose is located both in amorphous background and crystallites.

If our assumptions are valid, we could expect the following at an increase of amylose content in starches: 1) non-linear increase in the content of substance located in amorphous regions (C_{abg}, Fig. 3), and 2) after the first stage of hydrolysis, amylose content in crystallites of double and triple null line starches should be similar and significantly less in comparison with single null line starch. Indeed, in consideration of the error in the measurements, the data presented in Table 2 show that the changes in the C_{abg} observed at increase of amylose content can not be described by linear function. Unfortunately, the calibration used for determination of apparent amylose content [39] did not allow the calculation of amylose content in crystallites from triple null line starch. However, as can be seen from Table 2, the apparent amylose content in crystallites from single null line starch is 2.6 times larger than in crystallites from double null line starch, i.e. single null line starch contain 2.6 times more defects (amylose 'tie-chains') in comparison with double null line starch.

Thus, the data of acidic hydrolysis of starches from three different groups are in agreement with DSC data and confirm the hypothesis concerning different localization of amylose in starches from different groups. However, it is worth noting that there are conflicting data concerning the influence of amylose on rates of acidic hydrolysis. For example, investigations of maize and legume starches have shown that amylose content did not correlate with the initial rate of acidic hydrolysis [54, 56]. At the same time, studies of rice starches have shown that the susceptibility to acidic hydrolysis is inversely correlated with amylose content [57, 58]. Analysis of obtained results and previously published data [53–58] suggests that either our hypothesis is not universal for all starches or previous investigations did not take into account a possibility of different localization of amylose macromolecules within starch granules.

Figure 4 shows the DSC-traces of crystallites produced after acidic hydrolysis. The melting process of crystallites from triple null line wheat starch is a single endothermic transition, whereas the DSC-trace of crystallites from single and double null line starches shows two endothermic transitions describing melting of two ordered structures. One of them can be ascribed to melting of crystalline lamellae, while the second transition can be attributed to the dissociation of amylose–lipid complexes or the melting of V_h-type crystals [17, 44]. It is well known that in contrast to melting of starch crystals, the melting process of amylose–lipid complexes as well as V_h-type structures is reversible [17, 59]. Therefore it can be expected that during reheating of the samples of lintnerized starches from single and double null lines only one endothermic transition corresponding to the peak with higher melting temperature from the first scan should be observed. Indeed, this can be seen from the DSC-traces of reheated samples (Fig. 4). This means that crystalline lamellae of double and single null line starches contain amylose– lipid complexes or V_h-type ordered structures, while in crystalline lamellae of triple null line starch such structures are absent. Summing up, amylose macromolecules in crystallites or amylopectin clusters can be both in the form of amylose 'tie-chains' not contributing to the melting enthalpy and in the form of single helical complexes with starch lipids or in the form of ordered V_h-type structures [59]. Certainly, these conclusion are valid for Wx-B and Wx-AB wheat starches, but for wheat starches of other types an additional investigation is needed.

At the same time it should be noted, that the melting temperatures both for crystalline lamellae and

amylose–lipid complexes (Fig. 4) for lintnerized starches are lower than for native starches (Figs 1 and 2). This can be explained by decrease of the thickness of these structures during acidic hydrolysis. Estimation of thickness of crystals (Eqs (4) and (5)) shows that the L_{crl} value for lintnerized starches is 3.2 nm, while in native starches the mean L_{crl} value is 5.1 nm (Table 1). Additionally, taking into account that (*i*) melting temperature of amylose complexes with fatty acids depend on a length of hydrocarbon radical [60], (*ii*) melting temperature of complexes in crystallites of lintnerized starches (Fig. 4) is lower than those in native starches (Fig. 1), it can be supposed that within crystallites amylose forms complexes with lipid molecules possessing shorter hydrocarbon radicals compared to lipid molecules located within amorphous background.

Summarizing the data concerning thermodynamic melting parameters for crystalline lamellae of investigated starches and the data of acidic hydrolysis, it could be supposed that depending on the combinations of active GBSS I isoforms, amylose macromolecules are located in different regions of wheat starch granules. If active GBSS I isoenzymes are absent, amylose macromolecules are located, predominantly, in amylopectin clusters thereby playing the role of defects oriented along the lamella stack (starches of the first group). If the biosynthesis of amylose is controlled by only one synthase, amylose molecules are located, predominantly, in the amorphous background in unordered conformation (starches of the second group). If the biosynthesis of amylose is controlled by two synthases, amylose macromolecules can be arranged both in amylopectin clusters and amorphous background (starches of the third group).

It is possible to suppose that localization of amylose macromolecules within starch granule depends directly from the rate of its biosynthesis. When the biosynthesis rate is relatively high, the concentration of amylose macromolecules during formation of amylopectin cluster is also high. Both processes proceed simultaneously and amylose chains will be incorporated in amylopectin cluster producing crystalline defects. When the amylopectin biosynthesis rate is higher than of amylose, amylose molecules are forced out by growing amylopectin and are located mainly in the amorphous background. The amylose biosynthesis rate depends directly from the GBSS I isozymes activity. So it is quite clear that two Wx gene loci could produce more GBSS I enzyme than only one locus, the amylose biosynthesis proceeds quicker resulting in higher amylose content as well as its altered location within starch granules.

Additionally the data presented allow calculation the amylose content in crystallites of investigated wheat starches. One should return to Thomson–Gibbs' equation (Eq. (6)) and calculate the γ_i values using the following: T_m^0 (366.5 K), ΔH_m^0 (35.5 J g^{-1}) and ρ_{crl} (1.48 g cm^{-3}) for A-type spherolitic crystals [61] and L_{crl} (mean value=5.1 nm) from Table 1. The changes of the γ_i values *vs.* amylose content in starches are shown in Fig. 5a. It is worth noting that generally calculated γ_i values are in agreement with earlier published data for other wheat starches [25].

Since the γ_i value is mainly governed by the surface entropy that is proportional to the content of defects [26], an increase of the γ_i values at increasing amylose content in starches is an expected result. It is worth noticing that the character of the γ_i changes (Fig. 5a) is similar to observed changes of ΔC_p^{hydr} *vs.* amylose content (Fig. 2c) which, as above mentioned, reflects accumulation of defects in starches at increase of amylose content. It is an additional argument that increase of amylose content in starches leads to accumulation of defects in crystallites.

It is well known that starch is a semi-crystalline compound and its melting temperature is determined by imperfection of crystalline structures. As was shown above, such defects consist of amylose molecules located in crystallites. It means that the ob-

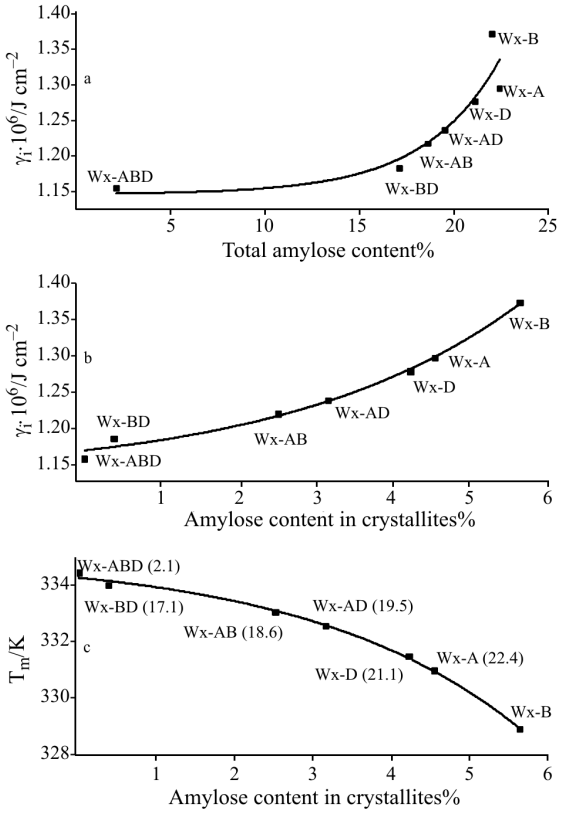

Fig. 5 Free surface energy of a – crystals face side for native wheat starches *vs*. total amylose content in starches, b – amylose content in crystallites and c – melting temperature of native wheat starches *vs.* amylose content in crystallites

served changes in melting temperature *vs.* total amylose content (Fig. 2b) should be presented as a function of amylose content in crystallites.

It could be supposed that dependence shown on Fig. 5a could be approximated by exponential function. Indeed, the results of such approximation (y_0=1.15·10^{-6}, A=4.7·10^{-10}, x_0=3.75, R=0.92) are reasonably good. Assuming that the function describing the changes of the γ_i *vs.* amylose content in crystallites is exponential, the values of amylose content in crystallites for Wx-B and Wx-AB starches are 5.4 and 2.1%, respectively, while for Wx-ABD the value is close to zero (Table 2), we can calculate parameters of the new function, assuming that this function should pass through three key points with γ_i (1.16·10^{-6} J cm^{-2}, 1.22·10^{-6} J cm^{-2}, 1.37·10^{-6} J cm^{-2}, Fig. 5a) and amylose content in crystallites (0, 2.1 and 5.4%), respectively. The result of this calculation is the following: y_0=1.31e^{-6}, A=3.66e^{-8}, x_0=3.0, (R=0.99). Using these parameters the values of amylose content in crystallites for other types of mutant starches could be easily calculated. The function describing the changes in γ_i *vs.* amylose content in crystallites is shown in Fig. 5b. Generally, calculated values of amylose content in crystallites are in agreement with data by Nakazawa and Wang [22] obtained during the study of Naegeli dextrins.

Obtained data allow representation of the observed changes in T_m values as a function of amylose content in crystallites (Fig. 5c). This function reflects the changes in melting temperature in native wheat starches according to Thomson–Gibbs' equation.

Conclusions

Application of different physico-chemical approaches to describe the changes in the thermodynamic melting parameters of native wheat starches with different combinations of GBSS I isoforms and different amylose content, as well as acidic hydrolysis of starches and thermodynamic properties of lintnerized starches allow one to suggest that an effect of different combinations of GBSS I isoenzymes acting in amylose biosynthesis is realized through different localization of amylose molecules within wheat starch granules, that reflects in structural and thermodynamic properties of native wheat starches as well as in the rates of acidic hydrolysis and amylose content in crystallites. For starches from double null line wheat types (one active GBSS I isoform), amylose is predominantly arranged within amorphous background or amorphous lamellae oriented transverse to the lamellar stack and only insignificant amounts are located within amylopectin clusters as amylose tie-chains. For starches from single null line wheat types (two active GBSS I isoforms), larger amounts of amylose are located in amylopectin clusters and influence on melting temperature of crystalline lamellae of starches in greater extent compared with triple and double null line starches. Irrespective of starch type, amylose tie-chains are arranged in crystalline lamellae of amylopectin clusters and can be considered as defects influencing on the thermodynamic melting parameters and the rates of acidic hydrolysis of native starches. It is worth noticing that changes in the melting temperature of starches, according to Thomson–Gibbs' equation, are determined by amylose content in crystallites (amylopectin clusters). Additionally, amylopectin clusters probably contain complexes of amylose with lipids, which have shorter hydrocarbon radicals compared to amylose–lipid complexes in amorphous background.

Acknowledgements

Experimental work was done with partial financial support from the Division of Chemistry and Science of Materials of Russian Academy of Sciences (Programme N6, 2003) and in the framework of joint grant from the Russian Ministry of Industry, Science and Technology and the Italian Ministry of Foreign Affairs (Executive routine of third mixed session of Russian–Italian commission on scientific and technical cooperation for 2003–2004 years signed in Rome by October 17, 2002. The appendix 3. No. 69AG2). The authors thank to Dr. H. Miura, Obihiro University of Agriculture and Veterinary Medicine, for providing wheat seeds (the wheat samples used are under the management of the National Bioresource Project-WHEAT, Japan) and to Dr. A. M. Kudryavtsev from Institute of General Genetics, Russian Academy of Sciences, for genetic consultations during preparation of this paper.

References

1 G. K. Moates, T. R. Noel, R. Parker and S. G. Ring, Carbohydr. Res., 298 (1997) 327.
2 P. Crochet, T. Beauxis-Lagrave, T. R. Noel, R. Parker and S. G. Ring, Carbohydr. Res., 340 (2005) 107.
3 V. P. Yuryev, N. K. Genkina and L. A. Wasserman, Zywnosc, 9 (2002), Supl., 155.
4 R. F. Tester, S. J. J. Debon, H. V. Davies and M. J. Gidley, J. Sci. Food Agric., 79 (1999) 2045.
5 N. K. Genkina, L. A. Wasserman and V. P. Yuryev, Carbohydr. Polym., 56 (2004) 367.
6 V. I. Kiseleva, N. K. Genkina, R. Tester, L. A. Wasserman, A. A. Popov and V. P. Yuryev, Carbohydr. Polym., 56 (2004) 157.
7 R. F. Tester, Int. J. Biol. Macromol., 21 (1997) 37.
8 J. P. Robin, C. Mercier, R. Charbonnière and A. Guilbot, Cereal Chem., 51 (1974) 389.
9 A. Imberty, H. Chanzy, S. Perez, A. Buleon and V. Tran, Macromolecules, 20 (1987) 2634.
10 A. Imberty, H. Chanzy, S. Perez, A. Buleon and V. Tran, J. Mol. Biol., 201 (1988) 365.

11 A. Imberty and S. Perez, Biopolymers, 27 (1988) 1205.
12 D. J. Manners, Carbohydr. Polym., 11 (1989) 87.
13 G. T. Oostergetel and E. F. J. Bruggen, Carbohydr. Polym., 21 (1993) 7.
14 P. J. Jenkins, R. E. Cameron and A. M. Donald, Starch/Stärke, 45 (1993) 417.
15 A. Buleon, P. Colonna, V. Planchot and S. Ball, Int. J. Biol. Macromol., 23 (1998) 85.
16 T. A. Waight, K. L. Kato, A. M. Donald, M. G. Gidley, C. J. Clarke and C. Riekel, Starch/Stärke, 52 (2000) 450.
17 V. P. Yuryev, L. A. Wasserman, N. R. Andreev and V. B. Tolstoguzov, Starch and Starch Containing Origins: Structure, Properties and New Technologies, Nova Science Publishers, New York 2002, p. 21.
18 V. P. Yuryev and L. A. Wasserman, Biochemistry and Chemistry: Research and Developments, Nova Science Publishers, New York 2003, p. 91.
19 G. Gérard, V. Planchot, A. Buleon and P. Colonna, Biopolymer Science: Food and non Food Applications, INRA, Paris 1999, p. 59.
20 N. Atichokudomchai, Z. Ao, D.-S. Suh and J. Jane, Abstract book of XII International starch convention, Krakow, Poland, June 14–18, 2002, p. 39.
21 E. Bertoft, Starch/Stärke, 56 (2004) 167.
22 Y. Nakazawa and Y.-J. Wang, Carbohydr. Res., 338 (2003) 2871.
23 Yu. I. Matveev, N. Elankin, E. N. Kalistratova, A. N. Danilenko, C. Niemann and V. P. Yuryev, Starch/Stärke, 50 (1998) 141.
24 Yu. I. Matveev, J. J. G. van Soest, C. Niemann, L. A. Wasserman, V. A. Protserov, M. Ezernitskaja and V. P. Yuryev, Carbohydr. Polym., 44 (2001) 151.
25 I. I. Bocharnikova, L.A. Wasserman, A.V. Krivandin J. Fornal, W. Błaszczak, V. Ya. Chernykh, A. Schiraldi and V. P. Yuryev, J. Therm. Anal. Cal., 74 (2003) 681.
26 V. A. Bershtein and V. M. Egorov, Differential Scanning Calorimetry of Polymers: Physics, Chemistry, Analysis, Technology, Ellis Horwood, New York 1994, p. 145.
27 A. Sharples, Introduction to Polymer Crystallization, Edward Arnold, London 1966, p. 55.
28 W. H. A. Mangalika, H. Miura, H. Yamauchi and T. Noda, Cereal Chem., 80 (2003) 662.
29 R. A. Graybosch, Trends Food Sci. Technol., 9 (1998) 135.
30 S. Chao, P. J. Sharp, A. J. Worland, E. J. Warham, R. M. D. Koebner and M. D. Gale, Theor. Appl. Genet., 78 (1989) 495.
31 T. Nakamura, M. Yamamori, S. Hidaka and T. Hoshino, Plant Breed., 111 (1993) 99.
32 H. Miura and S. Tanii, Euphytica, 72 (1994) 171.
33 H. Miura and A. Sugawara, Theor. Appl. Genet., 93 (1996) 1066.
34 S. G. Zeeman, T. Umemoto, W. L. Lue, P. Au-Yueng, C. Martin, A. M. Smith and J. Chen, Plant Cell, 10 (1998) 1699.
35 S. G. Ball, M. H. B. J. van de Wal and R. G. F. Visser, Trends Plant Sci., 3 (1998) 462.
36 G. E. Vandeputte and J. A. Delcour, Carbohydr. Polym., 58 (2004) 245.
37 H. Miura, M. H. A. Wickramasinghe, R. M. Subasinghe, E. Araki and K. Komae, Euphytica, 123 (2002) 353.
38 T. S. Gibson, V. A. Solah and B. V. J. McCleary, J. Cereal Sci., 25 (1997) 111.
39 K. Shibanuma, Y. Takeda, S. Hizukuri and S. Shibata, Carbohydr. Polym., 1994, 25, 111.
40 A. N. Danilenko, Ye. V. Shlikova and V. P. Yur'ev, Biophysics, 39 (1994) 427.
41 P. L. Privalov and N. N. Khechinashvili, J. Mol. Biol., 86 (1974) 665.
42 C. Gernat, S. Radosta, H. Anger and G. Damaschun, Starch/Stärke, 45 (1993) 309.
43 V. I. Kiseleva, A.V. Krivandin, J. Fornal, W. Błaszczak, T. Jelinski and V. P. Yuryev, Carbohydr. Res., 340 (2005) 75.
44 E. Nebesny, I. Kwasniewska-Karolac, J. Rosicka-Kaczmarek, Starch/Stärke, 57 (2005) 378.
45 Q. Liu, X. Lu and R. Yada, J. Therm. Anal. Cal., 79 (2005) 13.
46 K. Hayakawa, K. Tanaka, T. Nakamura, S. Endo and T. Hoshino, Cereal Chem., 74 (1997) 576.
47 K. Kohyama, J. Matsuki, T. Yasui and T. Sasaki, Carbohydr. Polym., 58 (2004) 71.
48 V. P. Yuryev, I. E. Nemirovskaya and T. D. Maslova, Carbohydr. Polym., 26 (1995) 43.
49 S. H. Yoo and J. Jane, Carbohydr. Polym., 49 (2002) 297.
50 B. Wunderlich, Macromolecular Physics, Vol. 1, Academic Press, New York 1973.
51 M. J. Gidley, personal communication, (1995), reference quoted from: P. J. Jenkins and A. M. Donald, Int. J. Biol. Macromol., 17 (1995) 315.
52 V. P. Yuryev, A. V. Krivandin, V. I. Kiseleva, L. A. Wasserman, N. K. Genkina, J. Fornal, W. Błaszczak and A. Schiraldi, Carbohydr. Res., 339 (2004) 2683.
53 N. Inouchi, D. V. Glover and H. Fuwa, Starch/Stärke, 39 (1987) 284.
54 C. G. Biliaderis, D. R. Grant and J. A. Vose, Cereal Chem., 58 (1981) 502.
55 H. Jacobs, R. C. Eerlingen, N. Rouseu, P. Colonna and J. Delcour, Carbohydr. Res., 308 (1998) 359.
56 E. Gérard, P. Colonna, A. Buleon and V. Planchot, Carbohydr. Polym., 48 (2002) 131.
57 A. D. Evers and B. O. Juliano, Starch/Stärke, 28 (1976) 160.
58 C. C. Maningat and B. O. Juliano, Starch/Stärke, 30 (1979) 5.
59 C. G. Biliaderis, Food Technol., 46 (1992) 98, 145.
60 S. Akuzawa, S. Sawayama and A. Kawabata, Biosci. Biotechnol. Biochem., 59 (1995) 1605.
61 M. A. Whittam, T. R. Noel and S. Ring, Food Polymers, Gels and Colloids, Royal Society of Chemistry, UK 1991, p. 277.

Received: September 5, 2005
Accepted: March 2, 2006
OnlineFirst: August 11, 2006

DOI: 10.1007/s10973-005-7319-y