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CHEMICAL PHYSICS OF POLYMER MATERIALS

Structural and Thermodynamic Characteristics of Potato Starches Depending on the Plant Genotype and Conditions of Their Cultivation

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Abstract—The creation of new highly productive forms of potato by genetic engineering methods raises the question about the quality of transgenic tubers, which first of all relates to the properties of important storage compound such as starch. The article presents the results of studies aiming to the analysis of morphology, structure, and thermodynamic parameters of starch extracted from the potato tubers expressing the *tms1* auxin biosynthesis gene from *Agrobacterium tumefaciens* and characterized by increased productivity in vitro. The transformed and control plants were cultivated in vitro on artificial sterile medium, as well as in vivo in the soil. The methods of scanning electron microscopy, wide-angle X-ray scattering, and differential scanning microcalorimetry were used in the work. It was established that the transformation of plants with the construction with the *tms1* gene under the control of the patatin gene promoter causes significant changes in a number of thermodynamic parameters of starch, first of all an increase in the thickness of crystalline lamella and melting temperature, which apparently reflects the increase in the structural ordering of the main starch fraction (approximately 90%) in the tubers of these transformants. Along with this, the effect of accumulating fractions with a less ordered structure in the starch composition, correlating with the ectopic expression of the *tms1* auxin biosynthesis gene, was established. At the same time, the B type of the polymorphic structure of starch remains unchanged. However, the detected changes mainly affected starch of the plants, cultivated in vitro. The starch of transgenic plants cultivated in the soil differs little in its basic characteristics from the starch of nontransgenic control plants.

Keywords: starch, *Solanum tuberosum* L., transgenic potato, *tms1* auxin biosynthesis gene, structure, morphology, thermodynamic parameters

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INTRODUCTION

Starch is the most common natural storage polysaccharide in plants, consisting of two types of polysaccharides: amylose and amylopectin. The peculiarities of the structure and the ratio of amylose and amylopectin in starches determine their general structure, as well as their physicochemical and functional properties. Starches of different botanical origins and their modifiers are widely used in different areas of industry as thickeners, stabilizing and gelling agents for the production of food and pharmaceutical products, as well as the components of new composite and biodegradable materials [1–5].

The tubers of potato (*Solanum tuberosum* L.) (the most important food, feed, and technical culture) are one of the most commercially significant sources of starch. The starch plays an important physiological role in the life cycle of the potato tuber. It is actively accumulated in a growing tuber, serves as a long-term storage of carbohydrates in a period of rest, and is remobilized during germination [6]. Starch metabolism in potato tubers was and remains the subject of numerous studies of regulating mechanisms and types of enzymes involved in the processes of germination and tuberization [7]. The molecular mechanisms underlying tuberization of this plant, as well as the processes of endogenous regulation of its growth and development, have still not been completely understood. Particularly, questions remain regarding the peculiarities of the structure and functions of starch during the ontogenesis of tubers.

Potato tuberization is regulated by many external and internal factors. Among the latter, phytohormones [8], determining the peculiarities of the course of plant morphogenesis and their resistance to unfa-

vorable environmental factors, play a significant role. The ability to manage these processes opens up the prospects of creating more productive and resistant varieties. This requires knowledge of the fundamentals of the effect of phytohormones at the molecular and cellular levels; in particular, understanding the peculiarities of their effect on the properties of valuable reserve deposits in tubers (first of all, starch) is important. The use of cultivated in vitro potato plants is one of the approaches used to study tuberization. A synchronous and fast response of plants to the standardized experimental conditions, as well as the possibilities of year-round studies of a large number of seedlings with varying conditions in a wider range than when grown in vivo, is an advantage of tuberization in vitro [9–11]. However, the results obtained when growing plants in artificial conditions do not always correspond to the results obtained in natural conditions [12]; therefore, the comparative analysis of plants grown in vitro and in vivo is always highly desirable.

Publications relating to the structural and thermodynamic peculiarities of potato tuber starches depending on hormonal status were not found in the literature. However, some studies demonstrated that the effect of auxin (1 mg L^{-1}) on single-node potato cuttings in vitro leads to an increase in the content of the starch in forming tubers and some increase in the size of starch granules [13]. This trend manifested itself even more in the potato line with the *rolB* transgen from the *A. tumefaciens* agrobacteria, which has an auxin-like effect. The starch of *rolB*-transgenic potato was thoroughly studied using modern methods [14]. Differences in physicochemical properties of the starch of *rolB*-transformants from the starch of the control plants, including the decrease in the thickness and melting temperature of the crystalline lamella, were established. These results were subsequently confirmed; in addition, it was found that the conditions in which the plants are grown (in vitro or in vivo) also affect the starch properties [12].

The aim of this work is to study the structure, morphology of the starch granules, and thermodynamic parameters of their melting from the tubers of potato plants cultivated in vitro and in vivo, including with a changed hormonal status. The studies were carried out with the starches extracted from the tubers of potato transformants ectopically expressing the agrobacterial *tms1* auxin biosynthesis gene [15]. This transgen was actively expressed in the tubers under the control of a tuber-specific promoter of the class I patatin gene (B33 promoter). It was established that the expression of the *B33::tms1* construction in potato plants grown in vitro causes an increase in the auxin level mainly in tubers, which led to an increase in the productivity (the amount and weight of tubers) of transformants compared with the control plants [15].

EXPERIMENTAL

Materials

Previously, transgenic potato plants of the Desiree variety (four independent lines) expressing the agrobacterial *tms1* auxin biosynthesis gene under the control of a tuber-specific promoter of the class I patatin gene were generated [15]. The transformants were vegetatively propagated by single-node cuttings and were grown in sterile tubes, where the tuberization was induced by the increased content (5%) of saccharose in the medium. Some of the obtained microtubers were planted in soil and placed (simultaneously with the control microtubers) in long-day conditions at the temperature of 22 to 24°C, where full-grown plants grew from them and formed tubers. The starch was extracted from the obtained tubers and microtubers according to the method [16].

Methods

Scanning Electron Microscopy

The structure of starch granules was characterized using scanning electron microscopy. Microphotographs of starch granules were obtained using a Mira3 LMU scanning electron microscope (Tescan, Brno, Czech Republic) at room temperature in the conditions of high vacuum and accelerating voltage 500 V.

X-Ray Analysis

The type of polymorphic structure was established using a wide-angle X-ray scattering method. The X-ray diffraction of starches was studied in the air–dry state using the Bragg–Brentano method on a HZG 4 diffractometer (Freiberger Präzisionsmechanik, Germany) [17, 18] using X-ray CuK_α-radiation, $\lambda = 1.542$ Å.

Differential Scanning Microcalorimetry

The thermodynamic parameters of melting 0.3 wt % aqueous dispersions of starches were determined using highly sensitive differential scanning microcalorimetry (DSC) on a DASM-4 microcalorimeter (Pushchino, Russia). The sample volume was 0.5 cm^3 in a closed cell. The measurements were carried out in the temperature range 20–100°C at a constant pressure of 2.5 MPa and heating rate 2°C/min. The excess heat capacity scale for each experiment was calibrated using the Joule–Lenz effect. Previously, it was demonstrated that there was no need in these conditions to take into account the thermal lag and sample processing time in the calorimetric cell [19]. As a comparison solution, deionized water obtained by using Millipore Direct-Q3 (Merck, Germany) filters with a resistivity 18.2 MOhm cm at a temperature 25°C was used for measurement.

The average values of the thermodynamic melting parameters of the crystalline lamellas of starches were

determined at least in three parallel measurements. The melting temperature value corresponded to the maximum of the heat capacity peak on the thermogram. The value of the experimental melting enthalpy corresponded to the area under the peak of the excess heat capacity curve as a function of temperature. The molar melting enthalpy (ΔH_m) was calculated per anhydroglucose residue (162 g/mole). The process of melting starch can be considered quasi-equilibrium in the first approximation [17–21]; this makes it possible to apply a one-stage melting model, in which the process of melting starch is described as an equilibrium reaction between the native and molten states.

The values of van't Hoff enthalpy (ΔH^{vH}) were calculated as previously described [17, 20] using the following equation:

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

where R is the universal gas constant; T_m is the melting temperature of starch crystalline lamellae; C_p is the maximum ordinate of the heat capacity peak on the thermogram; and $\Delta C_p^{\exp}$ is the difference in the heat capacity values between the molten and native states of starch dispersions.

The cooperative melting unit (v) was calculated as described in the works [19, 21]:

$$v = \Delta H^{vH}/\Delta H_m, \quad (2)$$

where ΔH_m is the experimental molar crystalline lamella melting enthalpy.

The thickness of the crystalline lamella ($L_{cr.l}$) was calculated on the basis of the following equation [21]:

$$L_{cr.l} = 0.35v, \quad (3)$$

where 0.35 nm is a projection of the anhydroglucose residue on the axis of the amylopectin double helix [22, 23].

The Peak Fit program (AISN Software Incorporated, Version 4) was used for deconvoluting the thermograms. The thermodynamic parameters corresponding to each thermodynamic transition were calculated [21].

Rheology of Gels

The rheological characteristics of 9% aqueous starch gels were studied on an Anton Paar MCR 302 rheometer (Austria) in the mode of oscillating sinusoidal oscillations. The prepared samples of starch gels were placed in a gap between the measuring surfaces representing a plane—plane (d = 25 mm). The measurements were carried out in the frequency range 0–400 rad/s at t = 20°C.

The 9% gel samples were prepared by dispersing a portion of dry starch in deionized water with continuous mixing for 5–7 min. The obtained suspension was heated to 95°C with constant mixing and incubated for 7 min with continued mixing. A part of the melt was placed in a cylindrical teflon cell with a diameter of 25 mm and height of 5 mm and incubated at 20°C for 24 h.

RESULTS AND DISCUSSION

Using the method of scanning electron microscopy, it was demonstrated that all the studied starches were characterized by the presence of granules of different sizes (both large and small) (Fig. 1). Granulometric analysis demonstrated that the expression of the *tms1* auxin synthesis transgene led to the formation of more granules of irregular or cubic form in starches compared with the starch from tubers of untransformed (UT) plants; the starch granules of transgenic plants are characterized by a greater portion of large granules. At the same time, the discovered regularities were manifested under both conditions of plant cultivation (in vivo and in vitro). Previously, similar results were observed for the starches from both untransformed and *rol*-transgenic plants [12, 24]. It should be noted that starch granules in the tubers in vivo are characterized by a larger size and larger portion of granules of irregular shape than in vitro, which is probably associated both with the physiological peculiarities of the plant, from which the starch is isolated, and the biological properties of chloroplast or amyloplast [25, 26].

The type of polymorphic crystal structure of starches was established by the method of X-ray scattering. Diffractograms of starches from untransformed tubers and transformed with the *tms1* gene tubers cultivated in vivo are given in Fig. 2. The type of crystal structure was determined by the position of the diffraction maxima (reflexes) on the diffractogram by the Bragg–Brentano method [27]. The positions of reflexes for the samples with a different degree of *tms1* auxin synthesis gene expression were observed at the same 2θ values that corresponded to the B-type polymorphic structure. It should be noted that all potato starches extracted from the tubers in vivo are also characterized by the same degree of crystallinity.

The DSC method, based on a comparison of the effect of 0.6 M KCl on the melting temperature of the starch crystalline lamellae, was used in the case of in vitro cultivation conditions for the comparative characteristics of the type of polymorphic structure of the starches from untransformed and transgenic plants [28].

It is known that under the influence of 0.6 M KCl, the melting temperature usually increases by 7–12 degrees compared with water dispersion in the case of the A-type starch polymorphic structure, while it increases only by 0.5–4 degrees in the case of the B-type polymorphic structure [29]. In our case, it was established for the A4-7 starch line in vitro (which is characterized by a high degree of *tms1* transgene expression [15]) that the melting temperature in the 0.6 M KCl solution

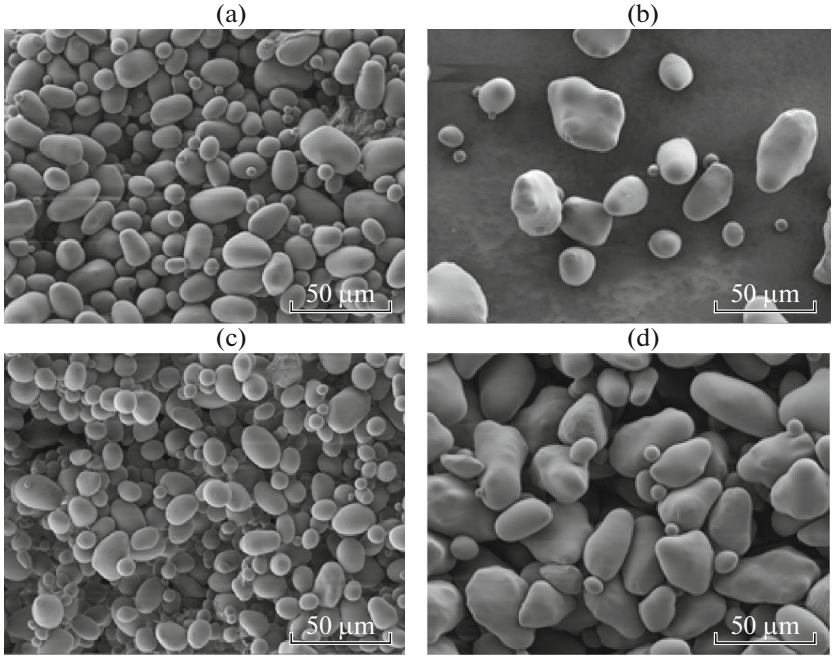

Fig. 1. Microphotographs of granules of the starch extracted from the tubers of UT plants (a, b) and from the tubers of *tms1*-transformants of potato (c, d) cultivated in vitro (a, c) and in vivo (b, d).

increased compared with water dispersion by 0.7 degrees, which is typical for the B-type polymorphic structure (no thermogram is given in Fig. 3).

Thus, the introduction of the *tms1* auxin synthesis gene does not cause in vivo and in vitro changes in the type of polymorphic structure of the starch in the tubers of potato plants. Previously, we obtained a similar result for the starches of potato expressing *rol*-transgenes [12, 14].

The thermodynamic melting parameters and thermograms of melting crystalline lamellae of the granules of potato starches extracted from untransformed and genetically modified (GM) plants expressing agrobacterial *tms1* auxin biosynthesis gene are given in Table 1 and in Fig. 3. The values of the melting temperature of the crystalline lamellae for starches from the transformants in vitro significantly increase with the simultaneous decrease in the value of the experimental starch melting enthalpy compared with the respective values for the starch from UT plants (Table 1). The calculation of the thermodynamic parameters according to the single-stage model demonstrated that the van't Hoff enthalpy changes in different directions; i.e., no significant change in this parameter is observed in either line. At the same time, the values of v and $L_{cr.\,l}$ parameters (Table 1) characterizing the cooperative melting unit and thickness of the crystalline lamellae increase significantly in the transformants in vitro (on the average by 24 and by 22%, respectively).

However, all of these changes in the characteristics are related to the starches isolated from plants that were cultivated in vitro. In the case of starches expressing the auxin synthesis transgen from the transformants in vivo, the temperature and melting enthalpy, as well as the thickness of the crystalline lamellae, barely changed compared with the corresponding values for the starch from UT plants (Table 1). These results are generally consistent with the previous observations that the starches of GM and UT plants grown in soil are more similar to each other compared with the starches of plants cultivated in vitro [12].

Taking into account that the thermograms of the studied starches are characterized by some asymmetry of the melting peaks, it can be assumed that this is a manifestation of the presence of more than one independent transition of crystal structures with different melting temperatures. The data on the deconvolution of the heat capacity curves according to the model of two independent transitions "all or nothing" are given in Table 2. An example of the deconvolution of the DSC thermogram is given in Fig. 3 for line A 1-2 in vivo (dashed lines on the curve *5*).

The data given in Table 2 show that two independent transitions caused by the melting of structures with different melting temperatures are manifested when melting the studied starches both from untransformed and transgenic plants. The presence of a low temperature transition in the case of starches from UT plants probably reflects the melting of the crystalline lamellae with a more defective structure than the main

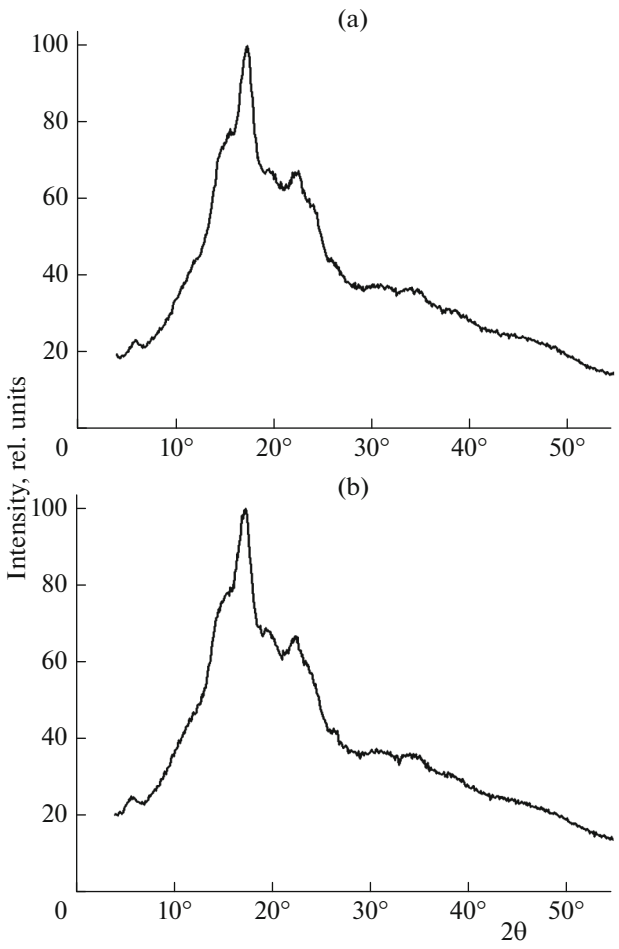

Fig. 2. Diffractograms of potato starches from UT tubers (a), tubers transformed with *tms1* auxin synthesis gene (b). Plants were cultivated in vivo.

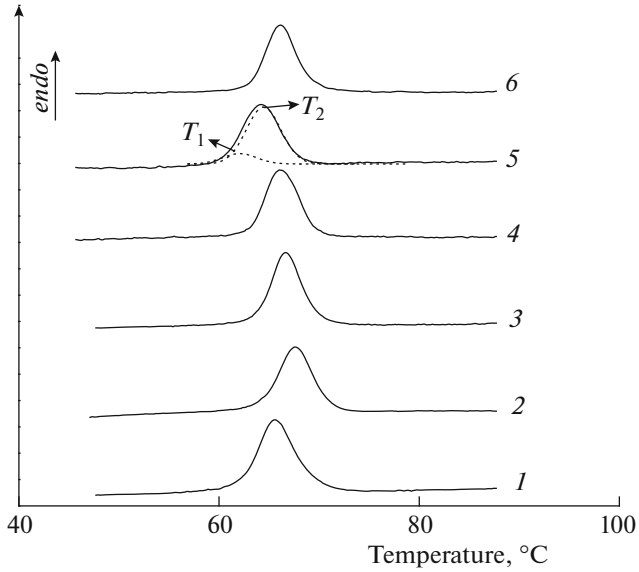

Fig. 3. DSC thermograms of melting of aqueous dispersions of starches with a concentration of 0.3 wt % extracted from the tubers of potato cultivated in vitro (*1*–*3*) and in vivo (*4*–*6*): (*1*, *4*) UT plants; (*2*, *5*) and (*3*, *6*) *tms1*-transformants of potato (independent A1-2 and A4-7) lines. Dashed lines on curve (*5*) are a result of deconvolution of DSC thermogram (T_1, low temperature transition; T_2, high temperature transition).

peak. It is known that the melting of crystalline lamellae begins with the destruction of defective regions in starch crystals such as the ends of the chain and loop segments [30].

The structural and thermodynamic parameters for each of transitions calculated according to the results of the deconvolution of thermograms are given in Tables 2 and 3. We note that the parameters of starches of UT plants (control) cultivated in vivo and in vitro differ significantly. Therefore, below we will talk about relative changes in the parameters of starches from transgenic plants compared with the parameters of starches from UT plants in these cultivation conditions.

The values of the α parameter representing the portion of the melting enthalpy of each of transitions 1 and 2 in the general experimental melting enthalpy are given in Table 2. It is obvious that in conditions of the

Table 1. Thermodynamic and structural parameters of melting of crystalline lamellae of starches from the tubers of untransformed (UT) and *tms1*-transformants of potato of independent A1-2 and A4-7 lines in vitro and in vivo

Cultivation conditions	Variant (line)	T_m, °C	ΔH_m, kJ/mol	ΔH^{vH}, kJ/mol	v, rel. units	$L_{cr.\,l}$, nm
in vitro	UT	64.2 ± 0.1	4.1 ± 0.1	53.3 ± 0.2	12.9 ± 0.1	4.6 ± 0.1
	A1-2	66.1 ± 0.1	3.3 ± 0.1	50.9 ± 0.1	15.4 ± 0.5	5.4 ± 0.1
	A4-7	65.9 ± 0.1	3.3 ± 0.1	53.9 ± 0.2	16.3 ± 0.1	5.7 ± 0.1
in vivo	UT	65.5 ± 0.1	4.0 ± 0.2	49.9 ± 1.5	12.7 ± 0.1	4.4 ± 0.1
	A1-2	63.7 ± 0.1	4.3 ± 0.1	49.4 ± 0.9	11.4 ± 0.3	4.0 ± 0.1
	A4-7	65.5 ± 0.1	4.2 ± 0.1	50.6 ± 1.0	10.0 ± 0.9	4.3 ± 0.3

T_m, melting temperature of crystalline lamellas; ΔH_m, experimental molar enthalpy of crystalline lamella melting; ΔH^{vH}, van't Hoff enthalpy; v, value of cooperative melting unit; $L_{cr.\,l}$, thickness of crystalline lamella.

Table 2. Results of deconvolution of melting thermograms of starches from the tubers of untransformed (UT) and *tms1*-transformants of potato of independent A1-2 and A4-7 lines in vitro and in vivo

Cultivation conditions	Variant	Low temperature transition		High temperature transition	
		T_{m1}, °C	α_1, %	T_{m2}, °C	α_2, %
in vitro	UT	60.8	7.8	65.1	92.2
	A1-2 line	63.8	9.2	67.0	90.8
	A4-7 line	63.2	12.1	66.1	87.9
in vivo	UT	62.6	8.9	65.6	91.1
	A1-2 line	61.4	15.2	64.8	84.8
	A4-7 line	65.1	17.8	65.5	82.2

T_{m1} and T_{m2}, melting temperatures of transitions 1 and 2; α_1 and α_2, portions of molar melting enthalpies of transitions 1 and 2 in general experimental molar melting enthalpy.

Table 3. Calculated as a result of deconvolution of thermograms structural and thermodynamic parameters of melting of crystalline lamellas of starch granules from the tubers of untransformed (UT) and *tms1*-transformants of potato of independent A1-2 and A4-7 lines in vitro and in vivo

Cultivation conditions	Variant	Low temperature transition			High temperature transition		
		$\Delta H_{m\ 1}$, kJ/mol	v_1, rel. units	$L_{cr.\ l\ 1}$, nm	$\Delta H_{m\ 2}$, kJ/mol	v_2, rel. units	$L_{cr.\ l\ 2}$, nm
in vitro	UT	0.32	1.0	0.4	3.8	11.9	4.2
	A1-2 line	0.30	1.4	0.5	3.0	14.0	5.0
	A4-7 line	0.40	2.0	0.7	2.9	14.3	5.0
in vivo	UT	0.35	1.1	0.4	3.6	11.6	4.0
	A1-2 line	0.65	1.7	0.6	3.6	9.7	3.4
	A4-7 line	0.75	1.8	0.8	3.5	8.3	3.6

$\Delta H_{m\ 1}$ and $\Delta H_{m\ 2}$, melting enthalpies of transitions 1 and 2; v_1 and v_2, values of parameters of melting cooperativeness; $L_{cr.\ l\ 1}$ and $L_{cr.\ l\ 2}$, critical thickness of crystalline lamella.

tms1 auxin synthesis gene expression, the portion of starch structures corresponding to the low temperature transition 1 increased compared with α_1 of the starch UT plants; moreover, the observed effect is typical for both cultivation conditions (in vitro and in vivo). The decrease in the temperature difference of transitions 1 and 2 in conditions of the *tms1* transgen expression is also typical for both cultivation conditions (Table 2).

The melting temperatures corresponding to both low and high temperature transitions rose during cultivation in vitro in the series of starches "UT plants–transgenic plants"; at the same time, the values of the cooperative melting unit, as well as of the thickness of the crystalline lamellae, also increased. The enthalpy of transition 1 changed in different directions, while the enthalpy of transition 2 decreased.

The dispersion of the experimental data increases during the cultivation in vivo (Table 3). There is a trend towards an increase in the temperature of transition 1, while the temperature of high temperature transition does not change. It can be also noted that under the conditions in vivo, the same trends towards an increase in the enthalpy of the low temperature transition 1, value of the cooperative melting unit, and thickness of the crystalline lamella are observed for both transitions, as for the cultivation conditions in vitro. The melting enthalpy of the high temperature transition 2 barely changes (Tables 2 and 3).

Thus, the introduction of the *tms1* auxin synthesis gene in potato plants causes no change in the type of the polymorphic structure of the starches in them under these experimental conditions but is accompanied by a change in the form and size of granules, especially, an increase in the portion of irregularly shaped granules. As it follows from the data on the deconvolution of thermograms, the expression of the *tms1* auxin synthesis gene is accompanied in vitro by a change in the thermodynamic and structural parameters of the crystalline lamellae of the main starch portion towards an increase in thickness and greater orderliness (≈90%), and at the same time by the accumulation of the structures melting at a lower temperature (≈10%) both in vitro and in vivo.

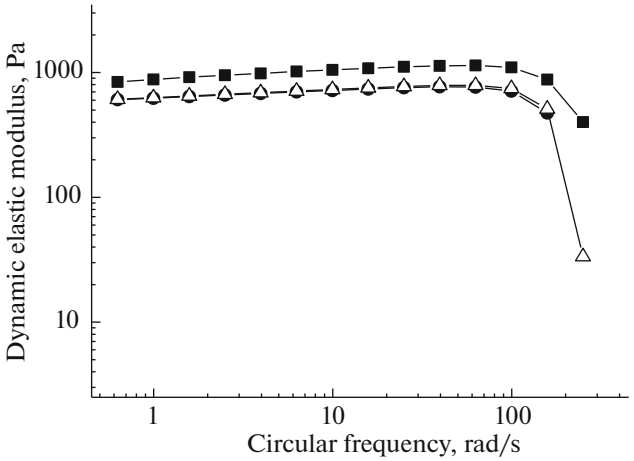

Fig. 4. Dependences of dynamic elastic modulus on circular frequency for potato starches from UT tubers (■), A1-2 (Δ) and A4-7 (●) lines cultivated in vivo.

To estimate the effect of the expressed *tms1* auxin synthesis gene on the viscoelastic properties of potato starch gels, the preliminary data on the rheology of 9% gels in water were obtained. The curves of the dependence of the dynamic complex modulus of elasticity on the circular frequency of the gels of starches isolated from UT plants and *tms1*-transgenic plants of two independent lines (A1-2 and A4-7 lines) cultivated in vivo are given in Fig. 4. It is obvious that the introduction of the *tms1* auxin synthesis gene in potato plants causes a decrease in the complex dynamic modulus of elasticity of the starch gel obtained from the transformants compared with the gel of the starch from UT plants. This result is correlated with the accumulation of starches of the fraction (≈10%) (when modifying the genome) characterized by less ordered structure compared with the starches from UT plants.

CONCLUSIONS

From these results on the study of granules of potato starches, it follows that the transformation of potato plants during the introduction of the *tms1* auxin synthesis gene in them has the following effects:

—it is accompanied by a change in the granulometric composition of starches (the portion of granules of an irregular shape and large size increases);

—it causes no change in the B-type polymorphic structure of starches;

—in vitro causes a change in the thermodynamic and structural parameters of the crystalline lamella melting of the main portion (≈90%) of the potato tuber starch (an increase in the temperature and value of the cooperative melting unit, growth of the thickness of the crystalline lamellae), as well as accumulation in the transformants of less ordered structures in starches;

—it reduces the viscoelastic properties of gels of the starch isolated from GM plants, which occurs in the conditions of the accumulation of the fraction with a less ordered structure in the composition of the starch compared with the main fraction of the lamellae.

Despite the significant quantitative differences, there are also common regularities in the change in the structural and thermodynamic melting parameters of starches in the *tms1*-transgenic plants cultivated in vivo and in vitro. From a practical point of view, this means that the expression of the *tms1* auxin synthesis gene in potato plants does not lead to significant violations in the formation and structure of the starch in the transformants.

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