Biochemistry (Moscow), Vol. 67, No. 2, 2002, pp. 227-232. Translated from Biokhimiya, Vol. 67, No. 2, 2002, pp. 271-277.

Biologically Active Oligosaccharides from Pectins of *Pisum sativum* L. Seedlings Affecting Root Generation

O. A. Zabotina[1]*, N. N. Ibragimova[1], A. I. Zabotin[1], O. I. Trofimova[1], and A. P. Sitnikov[2]

[1]Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, ul. Lobachevskogo 2/31, Kazan, 420503 Russia; fax: (7-8432) 387-577; E-mail: zabotin@mail.knc.ru
[2]Kazan State University, ul. Kremlevskaya 18, Kazan, 420503 Russia; fax: (8432) 380-994

Received April 6, 2001
Revision received June 7, 2001

Abstract—Two physiologically active oligosaccharide fractions were isolated from pectin of *Pisum sativum* L. cell wall after its partial acid hydrolysis. These fractions displayed stimulating and inhibiting effects on root formation in thin-layer explants. The subsequent separation of these fractions by gel permeation and anion-exchange chromatography resulted in fractions with effective concentrations two orders of magnitude lower than the concentrations of the initial fractions. The resulting oligosaccharides displayed their effect on the earliest stage of the rhizogenesis associated with formation of root primordias. The rhizogenesis-inhibiting fraction suppressed cell division by 30-50%. The stimulating fraction mainly contained fragments of xyloglucan and galactan, and the inhibiting fraction contained fragments of xyloglucan, galactan, and arabinan. The polymerization degrees of the stimulating and of the inhibiting oligosaccharides were 10-11 and 5-6, respectively.

Key words: *Pisum sativum* L., pectins, neutral oligosaccharides, root formation, growth, cell division

Partial hydrolysis of some polysaccharides of plant cell walls produces biologically active fragments called oligosaccharins that affect growth and organogenesis [1-3].

The rich variety of composition and structures of carbohydrate macromolecules that constitute plant cell walls suggests the existence of rather a large group of oligosaccharins of different composition and properties. This is supported by frequent reports on the detection of biologically active oligosaccharides. Some oligosaccharins were found in plant tissue *in vivo*, and this confirms the proposed existence of oligosaccharins as a new class of endogenous regulators [4, 5]. Physiologically active hemicellulose fragments have been obtained by the splitting of xyloglucan [5, 6], galactoglucomannan [7], and glucan [2], as well as pectin fragments obtained from homogalacturonan [8].

Pectin of dicotyledons consists of two main components, one of which is homogalacturonide consisting only of α-1,4-D-galacturonic acid residues and the other is a strongly branched polymer with the main chain of alternating residues of L-rhamnose and D-galacturonic acid and the side chains as the arabinans, galactans, and arabinogalactans bound to the main chain. Oligogalacturonide fragments are intensively studied as oligosaccharins, that can affect plant cell metabolism either by mediating the plant defensive reaction upon infection with pathogens [2] or by modulating the hormone-induced morphogenetic response of explants [9]. On the other hand, so far fragments of pectin molecules consisting of neutral monomers have not received any attention as a promising source of oligosaccharins, in spite of its varied monosaccharide composition.

A method has been developed for isolation of oligosaccharides from hydrolyzate of the cell wall pectin fraction of pea (*Pisum sativum* L.) seedlings. After mild acid hydrolysis, an oligosaccharide fraction was detected, which displays biological activity during rhizogenesis in thin-layer explants [10]. Soluble oligosaccharides with a similar biological activity were isolated from the cell juice of the same plants [11]. Therefore, it was suggested that pectin polysaccharides should be a source of free oligosaccharins that were found in the cell juice. The present work is an extension of studies on the composition and properties of the neutral pectin fraction prepared by us from pea seedlings that affects rhizogenesis in thin-layer explants.

MATERIALS AND METHODS

The oligosaccharide fraction was isolated from the cell wall pectin of 12-day-old pea (*Pisum sativum* L.)

* To whom correspondence should be addressed.

0006-2979/02/6702-0227$27.00 ©2002 MAIK "Nauka/Interperiodica"

seedlings, Bulat cultivar, grown on tap water at 23-25°C and at 12-h illumination regime.

The main steps in the plant material fractionation and isolation of separate oligosaccharide fragments are shown schematically in Fig. 1. The first stages of isolation of the fraction enriched with neutral oligosaccharides (IPN) have been described in detail [10, 12]. The IPN fraction was separated by gel-permeation chromatography on a column (100 × 2.6 cm) with P-4 Biogel (BioRad, Sweden) equilibrated with distilled water and thermostatted at 60°C; the elution was performed with distilled water at the same temperature at the rate of 0.3 ml/min. The total content of neutral sugars was determined in each fraction collected (0.6 ml) by the orcin–sulfuric acid method, and the presence of uronic monomers was assessed with the *m*-hydroxybiphenyl reagent [13]. The fractions were combined as shown in Fig. 2, concentrated, and desalted on a column with Sephadex G-10 (Pharmacia, Sweden).

The oligosaccharide fraction was further delicately separated by high performance anion-exchange chromatography using a Dionex chromatographic apparatus (Sunnyvale, CA, USA) with a CarboPac PA 100 column (25 × 4 cm) and pulse-amperometric detection. The rate of the eluent flow was 25 ml/min, 1/25 of this flow passing through the detector and the main flow synchronously collected in 2 ml in the fraction collector. The separation was performed in a concentration gradient of 0.1 M sodium acetate (from 0 to 40%) in 0.1 M NaOH. Each time a sample of 500 μl was injected and the chromatography time was no more than 40 min. The resulting fractions were immediately neutralized to pH 5.5-6.0 by automated addition of 5 M acetic acid. The fractions collected were combined correspondingly to separate peaks on the chromatograms (Fig. 3), desalted on G-10 Biogel, concentrated, and tested for the presence of biological activity.

To analyze the monosaccharide composition of the active fractions, samples of 0.2-0.5 mg of the dry material were hydrolyzed with 2 M trifluoroacetic acid for 1 h at 120°C, with 1 μM myo-inositol as the internal standard. The resulting hydrolyzates were dried in an air flow at 50°C until the complete removal of acid, and monosaccharides were converted to polyol acetates as described in [14]. The neutral monosaccharides were analyzed by gas–liquid chromatography on a glass column (3 m × 2 mm) filled with Chrom WAW (80-100 mesh) with 3% OV-275. The content of uronic acids was determined colorimetrically with *m*-hydroxybiphenyl [13].

The glycoside bonds in the oligosaccharide fragments were characterized using methylation [15] followed by hydrolysis of the methylated products with 2 M trifluoroacetic acid that finally resulted in the appropriate acetylated products. Gas–liquid chromatography of these products was performed using a capillary column (10 m × 0.32 mm) with 3% OV-225 and temperature program from 150 to 200°C with temperature increasing 2°C per min and with detection by mass spectrometry.

The test for biological activity was described in [3]. In detail: thin-layer explants of buckwheat hypocotyls were grown individually and aseptically in Petri dishes (40 mm) on a liquid hormone-free culture medium. Oligosaccharide fractions were added at concentrations

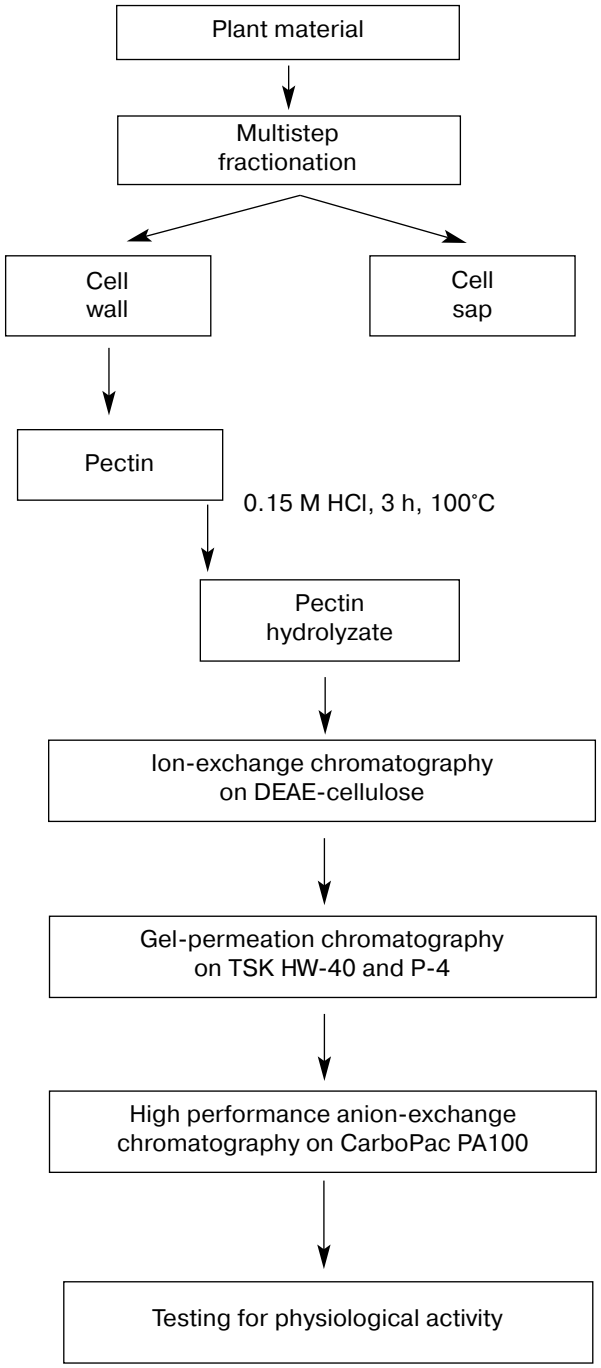

Fig. 1. Scheme of isolation of neutral oligosaccharide fragments from cell wall pectin of pea seedlings.

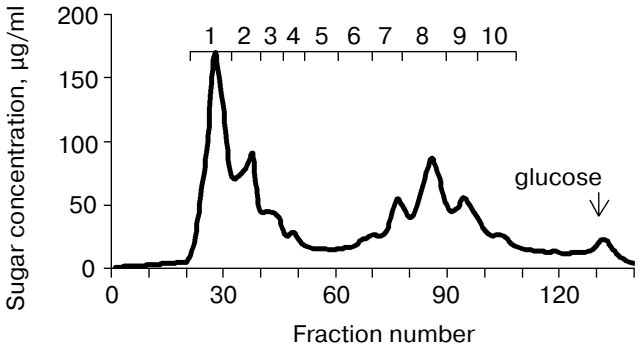

Fig. 2. Elution profile of the IPN oligosaccharide fraction by gel-permeation chromatography on a Biogel P-4 column (2.6 × 100 cm) with deionized water as the eluent (60°C). The fractions corresponding to separate peaks were combined as shown in the chromatogram. The column was calibrated with 5 kD dextran and glucose.

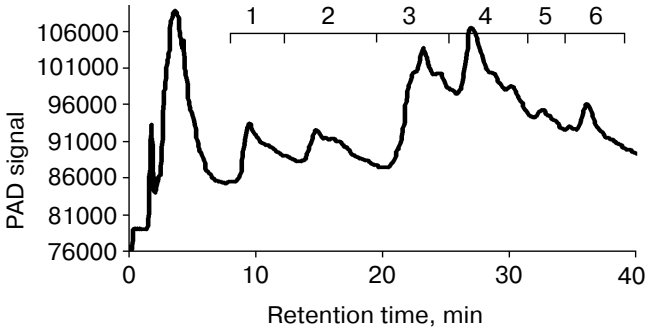

Fig. 3. Elution profile on preparative high performance anion-exchange chromatography of the oligosaccharide fraction IPN2 under gradient elution with sodium acetate solution of increasing concentration (0-40%) in 0.1 M KOH. The fractions corresponding to separate peaks were collected as shown in the chromatogram. PAD, pulse-amperometric detector.

from 10 to 0.01 μg/ml. Each fraction was independently tested three-to-five times on 30 separate explants.

The rate of the rootlet appearance on the explant surface was assessed by the method described in [16]. The rate was calculated by the formula:

$$E = \frac{N_1K_1 + N_2K_2 + \ldots N_iK_i}{N_i},$$

where E is the average rate (days) of the rootlet appearance onto the explant surface, N the number of explants producing rootlets on the day of numbering, K the time of the rootlet appearance (days), and i the final day of numbering.

Suspension cell culture of *Triticum timofeevi* Zhuk wheat was grown on medium containing 3% sucrose in the dark on a shaker (rotation rate 95 rpm) at 26°C. The medium of the experimental variants was supplemented with oligosaccharins at 0.01 μg/ml. The subculture period was 21 days. The cell growth in the culture was described by the increase in the raw weight and by the mitotic index. The cells were fixed with ethanol–acetic acid mixture (3 : 1) (Clark's fixer), then the cells washed free from the fixer were macerated with a mixture of glycosidases from *Trichoderma viridae* (Maxazime C. Gist Brocades, Netherlands). The cells were stained with orcein [17], prepared by the "pressurized drop" method [18], and examined with a Jenamed Histology microscope (magnification ×400). In each preparation more than 5000 cells were examined. The mitotic index was calculated as the ratio of number of divided cells to the total number of cells examined.

For histological analysis the thin-layer explants cultured for 5 days on the medium with oligosaccharin

Table 1. Effects of oligosaccharide fractions on root generation in thin-layer explants of buckwheat. An increase in the number of roots in the presence of the oligosaccharide fractions in the culture medium is expressed in percent to the control

Oligosaccharide fraction added	Changes in root number per explant, %				Weight of dry roots on 30th day of culture, %
	5-6 day	7-8 day	9-10 day	11-13 day	30 day
Control	100	100	100	100	100
IPN (1 μg/ml)	—	—	145	150	171
IPN 2 (1 μg/ml)	150	234	202	169	160
IPN 9 (1 μg/ml)	49	46	56	61	90
IPN 2/4 (0.01 μg/ml)	113	135	263	129	123
IPN 9/2 (0.01 μg/ml)	70	60	71	70	69

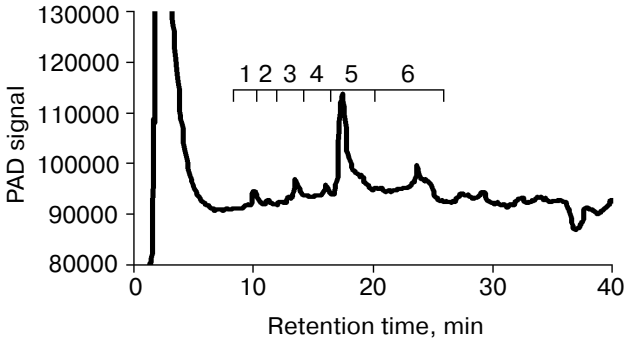

Fig. 4. Elution profile on preparative high performance anion-exchange chromatography of the oligosaccharide fraction IPN9 under gradient elution with sodium acetate solution of increasing concentration (0-40%) in 0.1 M KOH. The fractions corresponding to separate peaks were collected as shown in the chromatogram. PAD, pulse-amperometric detector.

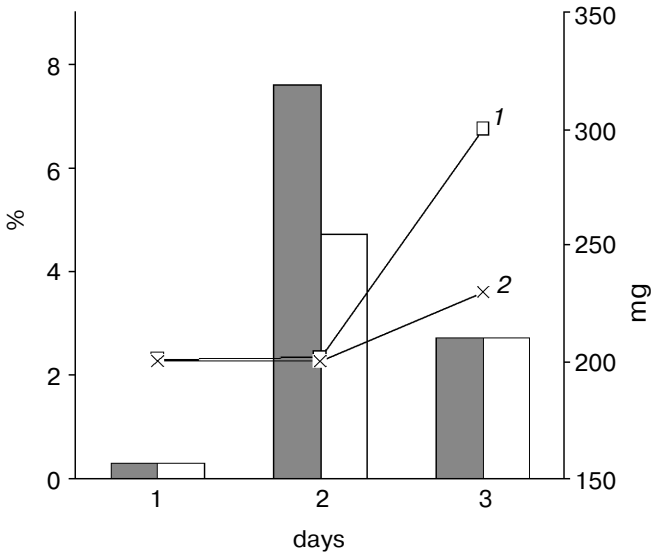

Fig. 5. Effect of the IPN9/2 oligosaccharide fraction on the development of suspension culture of wheat cells. The oligosaccharides were added into the culture medium at the concentration of 0.01 μg/ml at the beginning of a new passage. Columns show the mitotic index of the cells in percent (dark columns are the control, light columns are the experiment); lines show the cell weight in mg: *1*) control; *2*) experiment.

(0.01 μg/ml) or without it were fixed with Chamberlain's fixer (ethanol–acetic acid–formalin, 90 : 50 : 5) within 16 h. Then the preparations were poured into paraffin blocks, slices with a microtome were made, and stained with hematein as described in [19].

Active oligosaccharides were isolated four times; the elution profiles corresponded at all chromatographic separations, and compositions of the end products were similar.

RESULTS AND DISCUSSION

The first separation by gel-permeation chromatography on TSK HW-40 of the fraction enriched in neutral oligosaccharides (Fig. 1) revealed a physiologically active fraction IPN that stimulated root formation in thin-layer explants from hypocotyls of buckwheat [3]. Further separation of this fraction on P-4 Biogel (Fig. 2) produced ten oligosaccharide fractions, and only one of them (designated IPN2) displayed a similar although higher activity (Table 1). Concurrently, another fraction was found, IPN9, which suppressed the rhizogenesis. The significantly increased activity of the IPN2 fraction in comparison with the activity of the IPN fraction was due both to the increased relative content of the stimulating component and its separation from the component with the opposite effect.

The specific activities of the two fractions were significantly increased after their subsequent separation by high performance anion-exchange chromatography (Figs. 3 and 4). The fractions, that corresponded to separate peaks were tested in the same test system of the root-generating thin-layer explants. Fractions IPN2/4 and IPN9/2 obtained after the last procedure showed comparable activities at two orders of magnitude lower concentrations (Table 1). Thus, the final purification degree of the effectors was 1000-fold.

The adventitious root generation (from the stems cut off, from stem segments, or in thin-layer explants) occurs via some stages. After these stages the cells acquire the competence and ability for differentiation, which is terminated by the formation of primordia. Therefore, our next task was to determine the stage of the oligosaccharin involvement into the regulation of root generation.

The changes in the root weight noticed earlier under the influence of oligosaccharides can be associated with changes in both the root number and the rate of their development. Therefore, it was necessary to assess the effects of oligosaccharins on the rate of rootlet appearance on the explant surface. Results of these experiments

Table 2. Effects of the active oligosaccharide fractions on the average rate of rootlet appearance on the explant surface

Oligosaccharide fraction added	Rate of root appearance (*E*), days	
	control	experiment
IPN 2 (1.0 μg/ml)	7.5	6.5
IPN 2/4 (0.01 μg/ml)	6.8	6.8
IPN 9 (1.0 μg/ml)	6.6	7.1
IPN 9/2 (0.01 μg/ml)	6.7	6.8

Table 3. Monosaccharide composition (mole %) of the biologically active oligosaccharide fractions after each stage of the successive fractionation

Oligosaccharide fraction	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Xylose	Uronic acids
IPN	0.5	2.0	7.0	60.5	4.5	1.0	24.0
IPN 2	—	4.7	10.9	76.3	4.1	4.0	—
IPN 9	—	—	0.2	84.7	6.8	8.3	—
IPN 2/4	—	—	—	64.2	28.2	7.6	—
IPN 9/2	—	—	4.3	20.0	48.2	28.5	—

are presented in Table 2. The two active fractions obviously failed to affect this parameter. Thus, changes in the root number were not associated with the effect on the root growth rate but were determined by the number of root primordia formed. This was directly confirmed by histological analysis of the oligosaccharide effect on the root primordia formation in the explants. Thus, the rhizogenesis-inhibiting oligosaccharin IPN9/2 at 0.01 μg/ml nearly twofold decreased the primordia number (from 4.5 to 2.8 primordia per explant).

Table 4. Results of analysis of the oligosaccharide fractions resulted at the final stage of fractionation by methylation (mole % of the total amount of neutral sugars)

Replacement of monosaccharide residues	Oligosaccharide fraction added	
	IPN 2/4	IPN 9/2
t-Ara*	—	1.7
2-Ara	—	2.6
t-Xyl	—	2.5
4(2)-Xyl**	7.6	22.7
3,4-Xyl	—	3.3
t-Glc	8.7	33.6
4-Glc	10.1	7.3
t-Gal	4.3	10.2
4-Gal	6.5	—
6-Gal	50.0	8.8
3,6-Gal	3.4	—
4-Man	3.3	2.5
6-Man	6.1	4.8

* t means that the monosaccharide is terminal.
** Corresponding methylation products are determined together.

The oligosaccharide effect seems to be displayed during the earliest stages of rhizogenesis. According to current concepts, primordia formation includes a stage of active division of the differentiating meristematic cells [20]. The effect of oligosaccharide on the cell division was observed in the wheat root tip preparations, but most pronounced results were obtained in the experiments with wheat cell suspension culture. Data on mitotic index (MI) in the suspension of partially synchronized culture in its exponential phase indicated, that the IPN9/2 fraction decreased this parameter (Fig. 5).

The ability to inhibit root formation was earlier shown [8] for polygalacturonic acid degradation products, which contain only galacturonic monomers of the main chain of the pectin molecule. The monosaccharide compositions of all biologically active fractions obtained during the purification of the active components were analyzed, and the complete elimination of some polysaccharides (mainly of uronic acids) and their enrichment by xylose, glucose and galactose were shown (Table 3). The determination of glycoside linkages in the oligosaccharide molecules obtained at the final stage of purification of the active fractions (Table 4) suggests that the stimulating fraction should contain oligosaccharide fragments of xyloglucan and galactan and the inhibiting fraction should also contain fragments of xyloglucan, galactan, and arabinan.

Thus, we have found new oligosaccharins, which are liberated from the side chains of the neutral part of pectins and are involved in the early stages of root generation. These results extend the concepts on the biochemical stages of rhizogenesis.

The authors are grateful to Prof. A. Voragen (Vageningen Agricultural University, Netherlands) for the assistance in the analysis of the composition of the active oligosaccharide fractions. We are also grateful to researcher I. N. Smolenskaya of the Institute of Plant Physiology, Russian Academy of Sciences (Moscow), for her kind support with the suspension cell culture of winter wheat.

REFERENCES

1. Aldington, S., and Fry, S. C. (1993) *Adv. Botan. Res.*, **19**, 1-101.
2. Cote, F., and Hahn, M. (1994) *Plant Mol. Biol.*, **26**, 1379-1411.
3. Zabotina, O. A., Gurjanov, O. P., Ibragimova, N. N., Ayupova, D. A., and Lozovaya, V. V. (1998) *Plant Sci.*, **135**, 195-201.
4. Zabotina, O. A., Gurjanov, O. P., Ayupova, D. A., Beldman, G., Voragen, A. G. J., and Lozovaya, V. V. (1996) *Plant Cell Rep.*, **15**, 954-957.
5. Zabotina, O., Ayupova, D., Larskaya, I., Nikolaeva, O., Petrovicheva, G., and Zabotin, A. (1998) *Fiziol. Rast.*, **45**, 262-267.
6. Aldington, S., McDougall, G. J., and Fry, S. C. (1991) *Plant*, *Cell*, *Environ.*, **14**, 625-636.
7. Liskova, D., Auxtova, O., Kakoniova, D., Kubackova, M., Karacsonyi, S., and Bilics, L. (1995) *Planta*, **196**, 425-429.
8. Bellincampi, D., Cardarelli, M., Zaghi, D., Serino, G., Salvi, G., Gatz, C., Cervone, F., Altamura, M., Costantino, P., and de Lorenzo, G. (1996) *Plant Cell*, **8**, 477-487.
9. Tran Thanh Van, K., Toubart, P., Cousson, A., Darvill, A. G., Gollin, D. J., Chelf, P., and Albersheim, P. (1985) *Nature*, **314**, 615-617.
10. Zabotina, O. A., Gur'yanov, O. P., Malikhov, R. G., Ayupova, D. A., Beldman, G., Voragen, A. J. G., and Lozovaya, V. V. (1995) *Fiziol. Rast.*, **42**, 416-422.
11. Zabotina, O. A., Gur'yanov, O. P., Ayupova, D. A., Lozovaya, V. V., Beldman, G., and Voragen, A. (1997) *Biochemistry (Moscow)*, **62**, 845-849.
12. Zabotina, O., Ibragimova, N., Ayupova, D., Gurjanov, O., Lozovaya, V., Beldman, G., and Voragen, A. (1996) in *Pectin and Pectinases* (Visser, J., and Voragen, A., eds.) Elsevier Science, Ireland, pp. 693-701.
13. Ahmed, A. E., and Labavitch, J. M. (1977) *J. Food Biochem.*, **1**, 361-365.
14. Englyst, H. N., and Cummings, J. H. (1984) *Analyst*, **109**, 939-942.
15. Sandford, P. A., and Conrad, H. E. (1966) *J. Biochem.*, **5**, 1508-1517.
16. Reimers, F. E., and Illi, I. E. (1978) *Seed Germination and Temperature* [in Russian], Nauka, Novosibirsk, p. 104.
17. Stekol'shchikov, M. G., and Umylina, T. M. (1981) *Methodical Manual for Summer Practice in Genetics* [in Russian], Kazan State University, Kazan, p. 55.
18. Turkov, V. D., Guzhov, Yu. L., and Shelepina, G. A. (1988) *Studies on Plant Chromosomes in Problems of Selection*, *Cell Engineering*, *and Genetic Monitoring* [in Russian], People Friendship University, Moscow, p. 63.
19. Pausheva, Z. P. (1988) *Practical Work in Plant Cytology* [in Russian], Kolos, Moscow, p. 27.
20. Wightman, F., and Thimann, K. V. (1980) *Physiol. Plant.*, **49**, 13-20.