ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 7, pp. 861-865. © Pleiades Publishing, Ltd., 2010.
Published in Russian in Biokhimiya, 2010, Vol. 75, No. 7, pp. 978-983.

Recombinant Maize 9-Lipoxygenase: Expression, Purification, and Properties

E. V. Osipova, I. R. Chechetkin, Y. V. Gogolev, and N. B. Tarasova*

Kazan Institute of Biochemistry and Biophysics, Kazan Research Center, Russian Academy of Sciences, ul. Lobachevskogo 2/31, 420111 Kazan, Russia; fax: (843) 292-7347; E-mail: tarasova@mail.knc.ru

Received July 17, 2009
Revision received November 16, 2009

Abstract—Expression of maize 9-lipoxygenase was performed and optimized in *Escherichia coli* Rosetta(DE3)pLysS. The purity of recombinant protein obtained during Q-Sepharose and Octyl-Sepharose chromatographies in an LP system at 4°C was >95%. Maximum activity of the lipoxygenase reaction was observed at pH 7.5. Enzyme stability was studied at pH 4.5 to 9.5 and in the presence of different compounds: phenylmethanesulfonyl fluoride, β-mercaptoethanol, ammonium sulfate, and glycerol. HPLC and GC-MS analysis showed that enzyme produced 99% 9*S*-hydroperoxide from linoleic acid. 13-Hydroperoxide (less than 1%) consisted of S- and R-enantiomers in ratio 2 : 3.

DOI: 10.1134/S0006297910070072

Key words: maize 9-lipoxygenase, expression of 9-lipoxygenase, chromatography of 9-lipoxygenase, GC-MS analysis, HPLC

Lipoxygenases (EC 1.13.11.12) are key enzymes of the polyunsaturated fatty acids cascade in plants [1]. Lipoxygenases are monomeric proteins with molecular mass 94-104 kDa containing one atom of non-heme iron. Lipoxygenases are divided into (n + 2) type producing (13*S*)-hydroperoxides, (n – 2) type producing (9*S*)-hydroperoxides, and dual type based on specificity of dioxygenation of linoleic and linolenic acids [2]. More than 60 amino acid sequences of plant lipoxygenases are now known. For some of them, three-dimensional models have been obtained by X-ray analysis [3-6]. However, despite numerous sequencing and cloning of plant lipoxygenase genes, the biochemical characterization of enzymes is rare [7, 8], especially of the (n – 2) type. The mechanism of lipoxygenase catalysis is being discussed in the literature. Different approaches and methods [9-12] have failed to provide a clear answer to how regio- and stereospecificity of the reaction is controlled. Using pure lipoxygenase is an indispensable condition of such studies, since the spectrum and ratio of the products of the lipoxygenase reaction depend on the purity of the enzymes [13].

Abbreviations: GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria–Bertani (medium); PMSF, phenylmethanesulfonyl fluoride.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Expression of 9-lipoxygenase. Competent cells of *Escherichia coli* Rosetta(DE3)pLysS (Novagen, USA) were transformed by the pET30a vector with sequence of the 9-lipoxygenase gene kindly provided by N. Keller (University of Wisconsin, USA). Transformants were selected on solid LB (Luria–Bertani) medium with chloramphenicol (34 μg/ml) and kanamycin (50 μg/ml). Individual colonies were inoculated in 2 ml of LB medium containing kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), and glucose (0.01 M), grown for 7-8 h at 37°C and mild aeration (180 rpm) to A_{590} = 2.0. Then the culture was kept at 4°C for 8-10 h, and the cells were collected by centrifugation at 8000*g* for 5 min at 4°C. The pellets were suspended in 20 ml of LB and M9 media (1 : 1) supplemented with 0.1 M $FeSO_4 \cdot 7H_2O$, kanamycin (50 μg/ml), and chloramphenicol (34 μg/ml). The cells were grown to A_{590} = 2.0 and then transferred into 500 ml of fresh medium (1 : 50 v/v). The culture was grown to A_{590} = 0.6-0.7 and then induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM) and incubated for 16 h at 18-20°C under mild aeration (160 rpm). The cells were collected by centrifugation at 4000*g* for 15 min at 4°C and then washed with 0.05 M Na-phosphate buffer, pH 7.0, centrifuged, and suspended in the same buffer. The cells were disrupted using a French press

(Thermo Electron Corporation, USA) and centrifuged at 18,000*g* for 30 min at 4°C. The supernatant containing the target protein was used for subsequent work.

Purification of 9-lipoxygenase. Purification of the recombinant protein from the cell lysate of *E. coli* Rosetta(DE3)pLysS consisted of three stages: pretreatment, anion-exchange chromatography, and hydrophobic chromatography. The first stage consisted of protein precipitation by $(NH_4)_2SO_4$ (20-50% saturation at 4°C), followed by centrifugation at 18,000*g* for 20 min at 4°C. The precipitate was dissolved in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.01% Triton X-100 (buffer A), and dialyzed overnight against the same buffer. The dialyzed sample was applied to a column with Q-Sepharose (1.5 × 11 cm) equilibrated with buffer A using an LP chromatographic system (BioRad, USA). The protein was eluted with a linear gradient of NaCl (0-0.35 M) in the same buffer at a flow rate of 1 ml/min. The elution was monitored by absorption at 280 nm. Fractions containing lipoxygenase were collected and dialyzed for 18 h against 20% $(NH_4)_2SO_4$ in buffer A. Hydrophobic chromatography was carried out on Octyl-Sepharose (1.0 × 16.5 cm) equilibrated with 20% $(NH_4)_2SO_4$ in buffer A. The protein was eluted with a buffer with linearly decreasing concentration of $(NH_4)_2SO_4$ at flow rate 0.75 ml/min.

All manipulations with the 9-lipoxygenase were performed at 4°C. The purified 9-lipoxygenase was concentrated using ultrafiltration at 8000*g* in Vivaspin 20 tubes (5000 MWCO; Sartorius) at 4°C.

Protein content in samples. Protein was determined using Quick Start Bradford Dye Reagent (BioRad) and BSA as a standard.

Purity and molecular weight of the enzyme. Purity and molecular weight was determined by electrophoresis in 12% polyacrylamide gel in the presence of 0.1% SDS. The protein markers were β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), albumin (66.2 kDa), chicken egg ovalbumin (45.0 kDa), and carbonic anhydrase (31.0 kDa). Protein bands in gels were stained with Coomassie brilliant blue R-250 (Ferak, Germany).

Enzymatic activity. Lipoxygenase activity was determined by a method based on measuring the rate of accumulation of hydroperoxide, recorded by the absorption of the solution at 234 nm on a Lambda 25 spectrophotometer (Perkin-Elmer, USA). Standard analytical mixture (2 ml) contained 0.3 mM linoleic acid in 0.05 M Tris-HCl buffer, pH 7.5, previously saturated with oxygen for 3 min. The reaction was started by adding 2-10 μl of enzyme solution. The initial linear part of the kinetic curve was used to calculate the rate of enzymatic reaction using Cary Win Kinetics.

Determination of pH optimum and pH stability of 9-lipoxygenase. The pH dependence of the lipoxygenase reaction rate was studied with the following buffer systems: 0.05 M Na-acetate buffer (pH 4.5-5.5), 0.05 M Na-phosphate buffer (pH 6.5-7.5), 0.05 M Tris-HCl (pH 7.5-8.5), 0.05 M glycine-NaOH (pH 9.0 and 9.5). The pH stability of the 9-lipoxygenase enzyme was determined by preincubation in appropriate buffer solutions for 4 h at 4°C. Then the rate of the enzymatic reaction was determined as described above.

Analysis of products. Linoleic acid (0.5 mg) was incubated with 9-lipoxygenase in 0.05 M Na-phosphate buffer, pH 7.0, at 4°C for 30 min with continuous supply of oxygen. Then the reaction was stopped by adding glacial acetic acid to pH 4.5. Analysis of the reaction products was performed using normal-phase HPLC on two serially connected columns Separon SIX (3.2 × 150 mm), chiral-phase HPLC on a Chiralcel OD-H column (4.6 × 250 mm), and mass-selective Shimadzu QP5050A detector connected with a Shimadzu GC-17A gas chromatograph as described previously [14].

The table and figures show the data of typical experiments. The data were processed using the statistical *t*-test of Student. We discuss the differences statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Optimization of the expression conditions of 9-lipoxygenase (variation of growth temperature and time of addition of IPTG) in *E. coli* led to increased synthesis of the enzyme from 10 to more than 20% of the total cell protein. Addition of available iron ($FeSO_4$) to the growth medium increased the yield of active enzyme, although according to SDS-PAGE its content in cells remained unchanged. Lipoxygenases usually contain iron in the active state (Fe^{3+}) or inactive (Fe^{2+}) state [1, 2]. So, deficiency of iron could result in apoenzyme synthesis and in low initial activity of the enzyme. These techniques provided a source enriched with active enzyme.

The 9-lipoxygenase was isolated from lysate of *E. coli* Rosetta(DE3)pLysS by a combination of liquid chromatography methods: ion-exchange chromatography on Q-Sepharose and hydrophobic chromatography on Octyl-Sepharose in the LP chromatographic system at 4°C. Chromatographic profiles of 9-lipoxygenase are presented in Fig. 1. The lipoxygenase was eluted from a column of Q-Sepharose at 0.19-0.23 M NaCl (Fig. 1a) and Octyl-Sepharose column at 0% $(NH_4)_2SO_4$ (Fig. 1b). The results of three-stage purification of the enzyme are presented in the table. SDS-PAGE showed the presence of the major band of 9-lipoxygenase with a molecular mass of 96 kDa (Fig. 2), which corresponded to the theoretically calculated molecular mass of the recombinant enzyme. The purity of sample was >95%.

An important characteristic of any purified enzyme is its stability. Almost all lipoxygenases are extremely heat-labile proteins [2], so all manipulations with the protein were carried out at 4°C. The ability of 9-lipoxygenase to retain its activity in buffer solutions (pH 4.5-9.5)

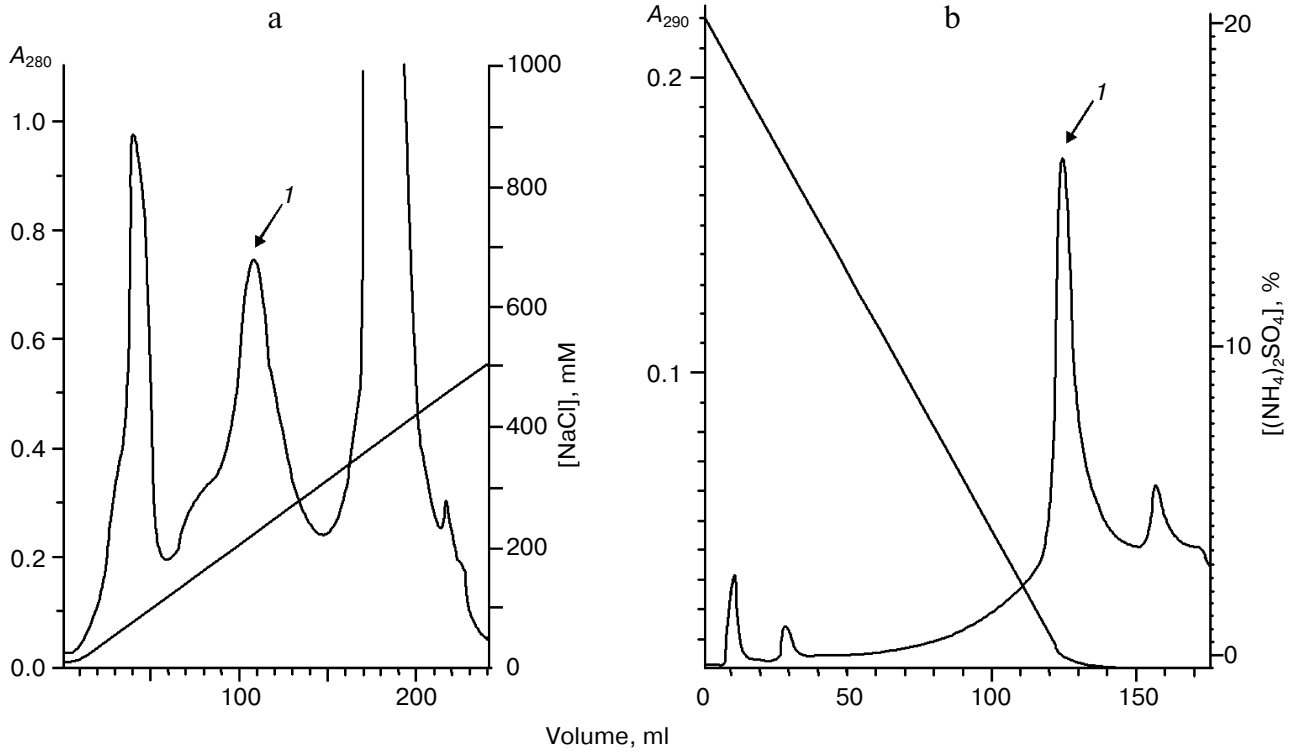

Fig. 1. a) Ion-exchange chromatography of 9-lipoxygenase on a column of Q-Sepharose. Volume of fractions, 3 ml. b) Hydrophobic chromatography of 9-lipoxygenase on Octyl-Sepharose column. Volume of fractions, 1.5 ml. *1*) Lipoxygenase peak.

was examined after 4 h preincubation of the enzyme in appropriate buffers. The enzyme retained the greatest activity (96%) in 0.05 M glycine-NaOH buffer, pH 8.5. In all other cases the residual activity of the enzyme was 20-30% of the original, which indicated to the instability of purified protein.

The dependence of the lipoxygenase reaction rate on pH is shown in Fig. 3. The maximum rate was recorded at pH 7.5. The optimal activity of the enzyme is consistent with the idea that 9-lipoxygenase usually functions in weakly acidic or neutral pH range [11, 15]. The difference between the optimal activity of lipoxygenase and its stability has been noted earlier, but such a wide pH range of enzymatic activity for 9-lipoxygenase is shown for the first time. Under acidic and alkaline conditions, the reaction yield rapidly plateaus. This pattern could be caused by protein denaturation at low and high pH values.

When exposed to 1 mM PMSF, the residual enzymatic activity after 1 h incubation with the inhibitor was only 5%. The mechanism of inhibitory action of PMSF on serine proteases is known. Analysis of the crystal structure of PMSF-treated human chymase showed that sulfonylation of Ser195 led to a significant change in the state of His57, which is a member of the catalytic triad of the enzyme, and the phenyl ring of the inhibitor was positioned at the entrance to the S1-cavity and might have contact with Phe191, Val213, and Gly216 [16]. The 9-lipoxygenase of maize contains 52 Ser residues, and three

Purification of 9-lipoxygenase

Purification stage	Protein, mg	Total activity, units	Specific activity, units/mg	Yield, %
Cell lysate	1570	207 000	132	100
$(NH_4)_2SO_4$ (20-50%) precipitation	435	170 000	390	82
Q-Sepharose	22	92 000	4180	45
Octyl-Sepharose	4.2	41 900	9970	20

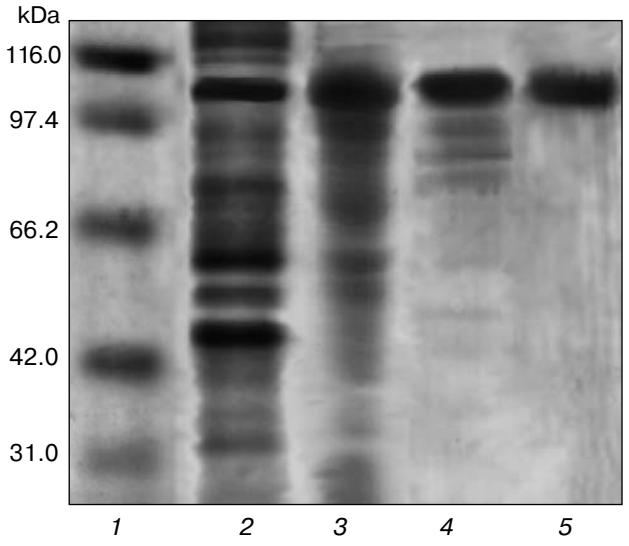

Fig. 2. Electrophoresis in 12% polyacrylamide gel with 0.1% SDS. Lanes: *1*) molecular weight markers; *2*) cell lysate; *3*) fraction of 20-50% $(NH_4)_2SO_4$ saturation; *4*) the protein from Q-Sepharose; *5*) the protein from Octyl-Sepharose.

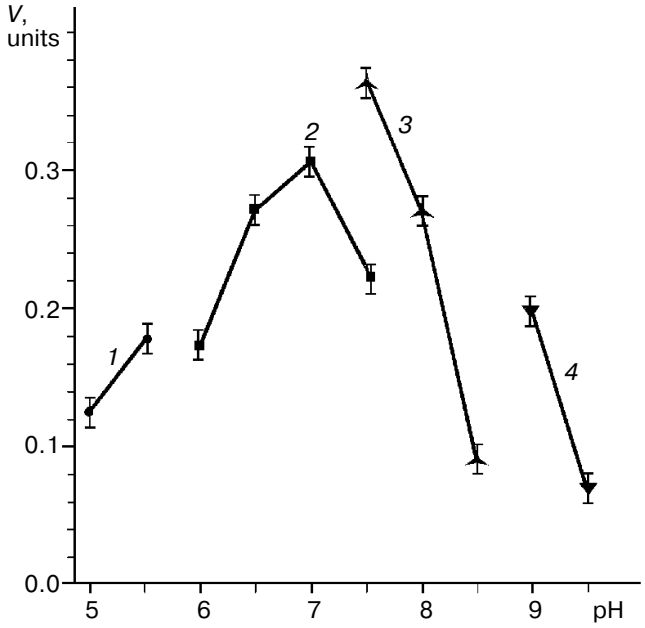

Fig. 3. Dependence of enzymatic reaction rate on pH: *1*) 0.05 M Na-acetate buffer; *2*) 0.05 M Na-phosphate buffer; *3*) 0.05 M Tris-HCl; *4*) 0.05 M glycine-NaOH.

of them are directly in the catalytic center of the protein. According to the three-dimensional computer model of the catalytic center, the side chains of Ser518 and His519, as well as Ser692, His695, and Ser863 are near each other. The comparison of amino acid sequences of plant lipoxygenases showed that His519 and His695 are highly conserved iron ligands. Thus, sulfonylation of serine by PMSF could lead to disruption of the catalytic center of lipoxygenase and consequently to the loss of catalytic activity.

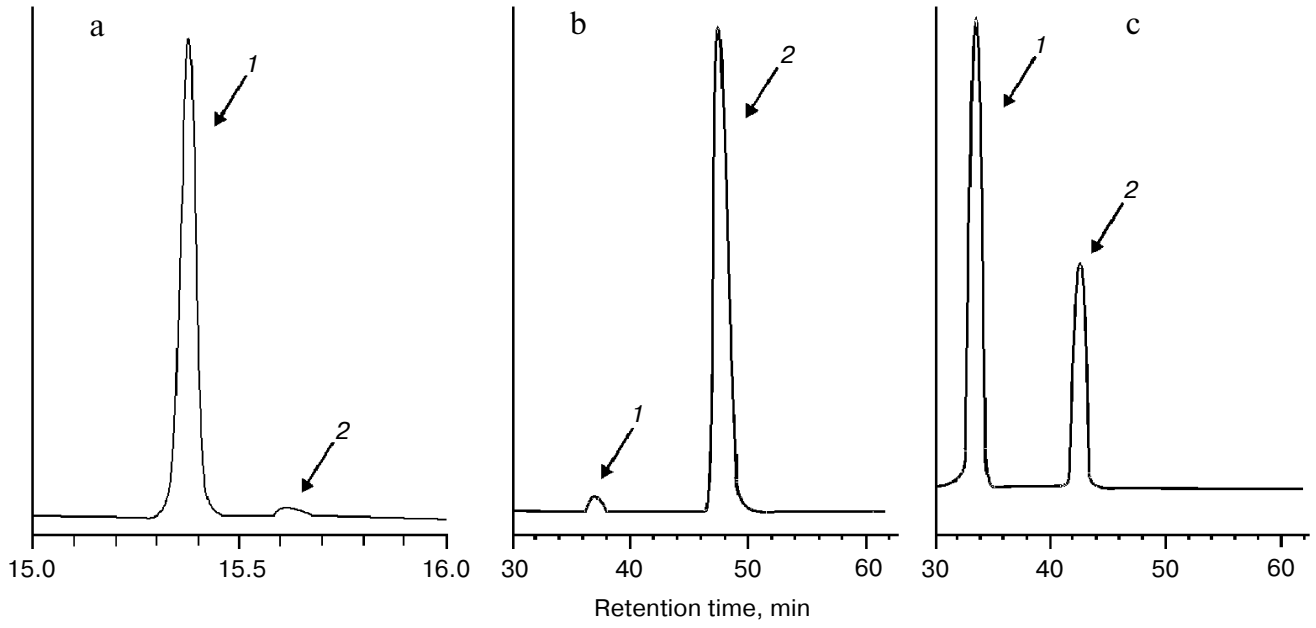

Fig. 4. a) GC/MS analysis of products of incubation of linoleic acid with 9-lipoxygenase at pH 7.0 (9-hydroperoxide (*1*) and 13-hydroperoxide (*2*) of linoleic acid). b) Chiral-phase HPLC of 9-hydroperoxide (9*R*-hydroperoxide (*1*) and 9*S*-hydroperoxide (*2*) of linoleic acid). c) Chiral-phase HPLC of 13-hydroperoxide (13*R*-hydroperoxide (*1*) and 13*S*-hydroperoxide (*2*) of linoleic acid).

Exposure of the protein to 1 mM β-mercaptoethanol also resulted in loss of 90% of its activity after 2 h. According to the computer model of a protein constructed on the basis of the homologous soybean lipoxygenase, Cys231 in the disordered chain of the C-domain and Cys699 in the α-helix of the C-domain, both situated in the catalytic center of the maize 9-lipoxygenase, are close together and likely form a cysteine bridge required for the catalytic properties of the enzyme.

Attempts to stabilize the 9-lipoxygenase in 0.01 M Tris-HCl buffer, pH 7.5, with glycerol (50%) or 2.3 M $(NH_4)_2SO_4$ gave positive results. The enzyme in glycerol solution frozen in liquid nitrogen and placed for storage at −86°C was active more than six months. In the presence of 2.3 M $(NH_4)_2SO_4$, the preparation retained its activity over six months at 4°C. However, when it was diluted in buffer systems we observed a disproportionate reduction in the activity of the enzyme due to the instability of protein in the diluted state [17].

According to current ideas about the mechanism of lipoxygenase catalysis, the specificity of lipoxygenase is determined by the substrate orientation in the active center [18, 19] and the presence or absence of a stereo-determinant near the site of dioxygenation. The methyl radical of an Ala residue on the surface of the substrate-binding cavity of the enzyme is usually considered as such stereo-determinant. Replacing Ala with Gly altered regio- and stereospecificity of the lipoxygenase reaction [20].

The analysis of products of dioxygenation of linoleic acid by GC/MS showed that at pH 7.0 linoleic acid was transformed mainly to 9-hydroperoxide (≥99%); 13-hydroperoxide yield was ≤1% (Fig. 4a). Using chiral-phase HPLC, it was revealed that 9-hydroperoxide was predominantly in S-configuration (Fig. 4b), while 13-hydroperoxide was a mixture of S- and R-enantiomers in the ratio 2 : 3 (Fig. 4c).

Thus, recombinant 9-lipoxygenase enzyme of maize with degree of purity >95% was obtained for the first time. The conditions required for isolation and long-term storage of the enzyme preparation in an active state were selected. The mechanism of fatty acid catalysis of 9-lipoxygenase using mutant forms of the protein requires further study.

The authors thank N. Keller (University of Wisconsin-Madison, USA) for providing the expression vector with cDNA of 9-lipoxygenase.

This work was supported by the Russian Foundation for Basic Research (grant 06-04-48430) and a grant of the President of the Russian Federation for State Support of Leading Scientific Schools (NSh-5492.2008.4).

REFERENCES

1. Feussner, I., and Wasternack, C. (2002) *Annu. Rev. Plant Biol.*, **53**, 275-297.
2. Liavonchanka, A., and Feussner, I. (2006) *J. Plant Physiol.*, **163**, 348-357.
3. Minor, W., Steczko, J., Stec, B., Otwinowski, Z., Bolin, J. T., Walter, R., and Axelrod, B. (1996) *Biochemistry*, **35**, 10687-10701.
4. Skrzypczak-Jankun, E., Amzel, L. M., Kroa, B. A., and Funk, M. O. (1997) *Proteins*, **29**, 15-31.
5. Youn, B., Sellhorn, G. E., Mirchel, R. J., Gaffney, B. J., Grimes, H. D., and Kang, C. (2006) *Proteins*, **65**, 1008-1020.
6. Gillmor, S. A., Villasenor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) *Nat. Struct. Biol.*, **4**, 1003-1009.
7. Wilson, R. A., Gardner, H. W., and Keller, N. P. (2001) *Mol. Plant Microb. Int.*, **14**, 980-987.
8. Boeglin, W. E., Itoh, A., Zheng, Yu., Coffa, G., Howe, G. A., and Brash, A. R. (2008) *Lipids*, **43**, 979-987.
9. Chechetkin, I. R., Osipova, E. V., Tarasova, N. B., Muhitova, F. K., Hamberg, M., Gogolev, Y. V., and Grechkin, A. N. (2009) *Biochemistry* (Moscow), **74**, 855-861.
10. Ruddat, V. C., Mogul, R., Chorny, I., Chen, C., Perrin, N., Whitman, S., Kenyon, V., Jacobson, M. P., Bernasconi, C. F., and Holman, T. R. (2004) *Biochemistry*, **43**, 13063-13071.
11. Hornung, E., Walther, M., Kuhn, H., and Feussner, I. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 4192-4197.
12. Schwarz, K., Walther, M., Anton, M., Gerth, C., Feussner, I., and Kuhn, H. (2001) *J. Biol. Chem.*, **276**, 773-779.
13. Chechetkin, I. R., Muhitova, F. K., Gogolev, Y. V., and Grechkin, A. N. (2007) *Dokl. RAN. Biokhim. Biofiz.*, **415**, 225-227.
14. Chechetkin, I. R., Blufard, A., Hamberg, M., and Grechkin, A. N. (2008) *Phytochemistry*, **69**, 2008-2015.
15. Butovich, I. A., and Reddy, C. C. (2001) *Biochim. Biophys. Acta*, **1546**, 379-398.
16. McGrath, M. E., Mirzadegan, T., and Schmidt, B. F. (1997) *Biochemistry*, **36**, 14318-14324.
17. Scopes, R. (1985) *Methods for Protein Purification* [Russian translation], Mir, Moscow.
18. Egmond, M. R., Vliegenthart, J. F. G., and Boldingh, J. (1972) *Biochem. Biophys. Res. Commun.*, **48**, 1055-1060.
19. Gardner, H. W. (1989) *Biochim. Biophys. Acta*, **1001**, 274-281.
20. Coffa, G., and Brash, A. R. (2004) *Proc. Natl. Acad. Sci. USA*, **101**, 15579-15584.