Biochimica et Biophysica Acta, 1074 (1991) 443–447

ADONIS 030441659100213E

BBAGEN 20283

Rapid Report

Improved purification of 12-lipoxygenase from rat basophilic leukemia cells and conditions for optimal enzyme activity

Eric M.M. Van der Donk, Gerald R. Dubois, Jan Verhagen, Gerrit A. Veldink and Johannes F.G. Vliegenthart

Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, Utrecht University, Utrecht (The Netherlands)

Key words: 12-Lipoxygenase; 13-Hydroxyoctadecadienoic acid; (Rat basophilic leukemia cell)

12-Lipoxygenase from rat basophilic leukemia cells was purified about 300-fold by protein-HPLC in a single run. Maximal 12-lipoxygenase activity was observed at pH 7.5, while the enzyme became almost inactive at pH 6 and 9. Although Ca^{2+} was not essential for 12-lipoxygenase activity, the partially purified enzyme was stimulated approx. 2-fold in the presence of 0.1–5.0 mM Ca^{2+}. Contrary to 5-lipoxygenase from RBL-1 cells, 12-lipoxygenase was not inactivated by preincubation with Ca^{2+} for 1–10 min, nor was it stimulated by 0.1–10 mM ATP.

Rat Basophilic Leukemia (RBL-1) cells have been shown to possess 12-lipoxygenase activity [1–3]. Recently, we reported the separation of 12-lipoxygenase from 5-lipoxygenase by protein-HPLC [4]. The enzyme was shown to convert arachidonic acid into 12(*S*)-HETE and linoleic acid into 13(*S*)-H(P)OD. Optimal 12-lipoxygenase activity was observed at 10 °C, while at 37 °C 12-lipoxygenase was very rapidly inactivated by low concentrations (10–100 nM) of its product, hydroperoxy fatty acid. Linoleic acid was found to be a substrate for 12-lipoxygenase but not for 5-lipoxygenase from RBL-1 cells. Therefore, 12-lipoxygenase activity in the cytosolic fraction can be studied without interference of simultaneously formed 5-lipoxygenase products by using linoleic acid as a substrate. 12-Lipoxygenase from RBL-1 cells resembles 12-lipoxygenases from bovine and porcine leukocytes, which can also utilize linoleic acid as a substrate [5–7], in contrast to 12-lipoxygenase from bovine and human platelets [8].

RBL-1 cells (Flow Laboratories, U.K.) were grown in stationary tissue culture flasks in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% foetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and amphotericin (10 μg/ml). Cells were collected, centrifuged (10 min, 2000 × *g*, room temperature), and washed with phosphate-buffered saline (PBS, 0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4). After cell disruption 12-lipoxygenase was partially purified from the 20000 × *g* cell supernatant as described before [4] with one modification; no phosphatidylcholine (PC) was added to buffers. Briefly, cell supernatant was brought to 50% saturation with 100% saturated ammonium sulfate (pH 7.1) and the protein precipitate was resuspended in buffer B (20 mM Tris-HCl (pH 7.1), 50 mM NaCl, and 5% ethylene glycol), dialyzed, centrifuged, and injected onto a TSK DEAE-5PW anion-exchange HPLC column (7.5 × 75 mm) with a poly-ol Si 300 gel filtration HPLC guard column, preequilibrated at 0 °C with buffer B at a flow rate of 0.5 ml/min. The highest 12-lipoxygenase activity eluted in the pass-through fractions. Protein concentrations were determined according to Bradford [9].

The enzyme assay was carried out in PBS (cell supernatant) or buffer B (purified fractions). The amount of 13-H(P)OD formed upon incubation with linoleic acid (99%, Fluka AG, Buchs, Switzerland) during 30 min was used as a measure of lipoxygenase activity. The enzyme assay was performed at 10 °C and pH 7.5 in a final volume of 500 μl containing 60 μM linoleic acid, 1 mM Ca^{2+}, and 5% ethylene glycol, unless otherwise indicated. Incubations were stopped by the addition of 500 μl methanol and formed 13-HPOD was reduced to 13-HOD by the addition of reduced glutathione (final concentration 5 mM; Sigma

Abbreviations: H(P)ETE, hydro(pero)xyeicosatetraenoic acid; H(P)OD, hydro(pero)xyoctadecadienoic acid; PBS, phosphate-buffered saline (0.9% (w/v) NaCl in 8.6 mM phosphate buffer, pH 7.4); RBL, Rat Basophilic Leukemia; PC, phosphatidylcholine.

Correspondence: J.F.G. Vliegenthart, Bijvoet Center for Biomolecular Research, Department of Bio-Organic Chemistry, University of Utrecht, Padualaan 8, NL-3584 CH Utrecht, The Netherlands.

Chemicals, St. Louis, MO, U.S.A.). Prostaglandin B_2 (Sigma) was added as an internal standard for HPLC analysis. 13-HOD was extracted with octadecyl solid-phase extraction columns (Baker, Deventer, The Netherlands) and analyzed with reversed-phase HPLC as described before [4]. The identity of 13-HOD was confirmed by ultraviolet detection and by co-elution with 13-HOD prepared by incubation of linoleic acid with soybean lipoxygenase-1 at pH 9.0 [10]. 13-HOD was quantified by relating peak areas to that of the internal standard prostaglandin B_2. Molar absorption coefficients used are: 28650 M^{-1} cm^{-1} at 280 nm for prostaglandin B_2 and 25000 M^{-1} cm^{-1} at 237 nm for 13-HOD [11].

12-Lipoxygenase from RBL-1 cell supernatant was partially purified in a single run, using a TSK DEAE-5PW anion-exchange HPLC column with a poly-ol Si 300 gel filtration HPLC guard column. In our previous report [4] sonicated PC (type III-E; Sigma) was added to buffer B (final concentrations: 100 μg/ml during dialysis and 20 μg/ml during elution) to improve recovery of 5-lipoxygenase activity [12,13]. Following this procedure, purification of 12-lipoxygenase was only about 30-fold (Table I). However, when sonicated PC was left out from buffer B, recovery of 12-lipoxygenase activity was strongly improved, resulting in an almost 300-fold purification (Table I). To investigate if part of the 12-lipoxygenase was bound tightly to the column in the presence of sonicated PC, a linear gradient of 50–400 mM NaCl in buffer B was applied to the column to elute 5-lipoxygenase [4], followed by an injection of 1 ml of a 2 M NaCl solution. However, the resulting protein eluate did not contain any 12-lipoxygenase activity. In order to exclude that a direct inactivation of the enzyme by sonicated PC was involved, cell supernatant (Fig. 1A) or partially purified enzyme (Fig. 1B) was preincubated for 5 min with sonicated PC and subsequently incubated with linoleic acid. In both cases only a slight reduction in enzyme activity was observed at the concentration of sonicated PC previously used in elution buffer. Since both profiles seem to be similar, this slight reduction in enzyme

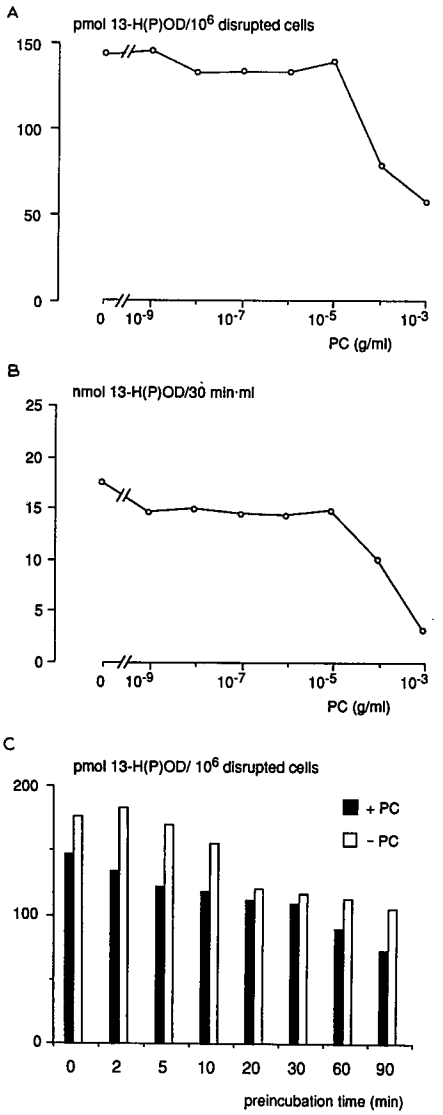

Fig. 1. Effect of added sonicated PC on 12-lipoxygenase activity. 20000× g Supernatant of RBL-1 cells (A) or partially purified 12-lipoxygenase (B) was incubated with 60 μM linoleic acid for 30 min at 10 °C in the presence of 1 mM Ca^{2+}. (C) Protein precipitate of 20000× g supernatant was preincubated without sonicated PC or with 100 μg/ml sonicated PC, diluted to 20 μg/ml sonicated PC, and incubated with 60 μM linoleic acid for 30 min at 10 °C in the presence of 1 mM Ca^{2+}. Circles and bars represent mean values of duplicate experiments with one enzyme preparation.

TABLE I

Partial purification of 12-lipoxygenase from RBL-1 cells with and without sonicated PC being added to buffers

Fractions were assayed for 12-lipoxygenase activity for 30 min at 10 °C and pH 7.5 in the presence of 60 μM linoleic acid, 1 mM Ca^{2+}, and 5% ethylene glycol. The reaction mixtures were analyzed by reversed-phase HPLC as described in [4].

Sample	Total protein (mg)	Total activity (nmol)	Specific activity (nmol/mg)	Purification (-fold)	Yield (%)
20000× g supernatant	81.8 (77.7)	28.9 (30.1)	0.35 (0.39)	1 (1)	100 (100)
0–50% precipitate	50.1 (45.5)	26.2 (28.6)	0.52 (0.63)	1.5 (1.6)	91 (95)
DEAE ion-exchange pass-through					
Fraction 10–16 min	0.296 (0.337)	0.89 (18.8)	3.02 (55.6)	8.5 (143.7)	3.1 (62)
Fraction 12–14 min	0.041 (0.050)	0.42 (5.4)	10.17 (108.4)	28.8 (280.8)	1.4 (18)

Values given in parentheses are those obtained in the absence of sonicated PC.

activity may be caused by incorporation of linoleic acid into PC aggregates and/or micelles, thereby reducing the amount of substrate available for the enzyme. However, this can not explain the substantially lower recovery of 12-lipoxygenase activity observed when sonicated PC is added during purification. To investigate a possible time-dependent inactivation of 12-lipoxygenase by PC during dialysis and subsequent centrifugation, the protein precipitate was preincubated at 100 μg/ml sonicated PC for various time periods and diluted to 20 μg/ml sonicated PC prior to enzyme assay (Fig. 1C). After preincubation with sonicated PC for 90 min a decrease in 12-lipoxygenase activity of about 50% was observed. However, after 90 min in the absence of sonicated PC, 12-lipoxygenase activity was decreased to the same extent. Therefore, the recovery of 12-lipoxygenase activity after column elution seems to be decreased substantially by the presence of 20 μg/ml sonicated PC in the elution buffer. It remains unclear what sort of interaction during column elution causes this decrease.

At 10 °C 12-lipoxygenase was found to have maximal activity at pH 7.5, while the enzyme became almost inactive at pH 6 and 9. A similar profile was reported for 5-lipoxygenase from RBL-1 cells [14] and the same pH optimum was also reported for 12-lipoxygenases from porcine leukocytes [7] and from bovine leukocytes and platelets [15]. Only 12-lipoxygenase from human uterine cervix was reported to have a pH optimum of 6.5 [16].

It is known that when RBL-1 cells are lysed in the presence of 0.05–10 μM Ca^{2+}, 5-lipoxygenase becomes associated with the particulate fraction, thereby losing its activity [17]. Addition of EGTA prior to cell lysis prevents this association. Moreover, addition of EDTA prior to cell lysis improves recovery of 5-lipoxygenase activity in cell supernatant [1,12], probably by binding Ca^{2+} from endogenous sources and/or by inhibiting metal ion-dependent proteolysis [13]. Although 5-lipoxygenase does require Ca^{2+} for optimal activity [1,13], in cell supernatant the enzyme was shown to become inactivated rapidly by preincubation with 1 mM Ca^{2+}. A decrease in activity of 90% was observed within 1 min [17].

Our experiments confirm these observations for 5-lipoxygenase from RBL-1 cells (data not shown). By contrast, we did not observe a decrease in 12-lipoxygenase activity after preincubation with 1 mM Ca^{2+} for 1–10 min nor an improved recovery of 12-lipoxygenase activity by adding EDTA prior to cell lysis.

To further investigate Ca^{2+} dependence of 12-lipoxygenase activity, cell supernatant was incubated with linoleic acid at increasing Ca^{2+} concentrations. We found that 12-lipoxygenase was stimulated about 2-fold at 0.2–5.0 mM Ca^{2+} (Fig. 2A). As the enzyme was also found to be active in the absence of added Ca^{2+}, it

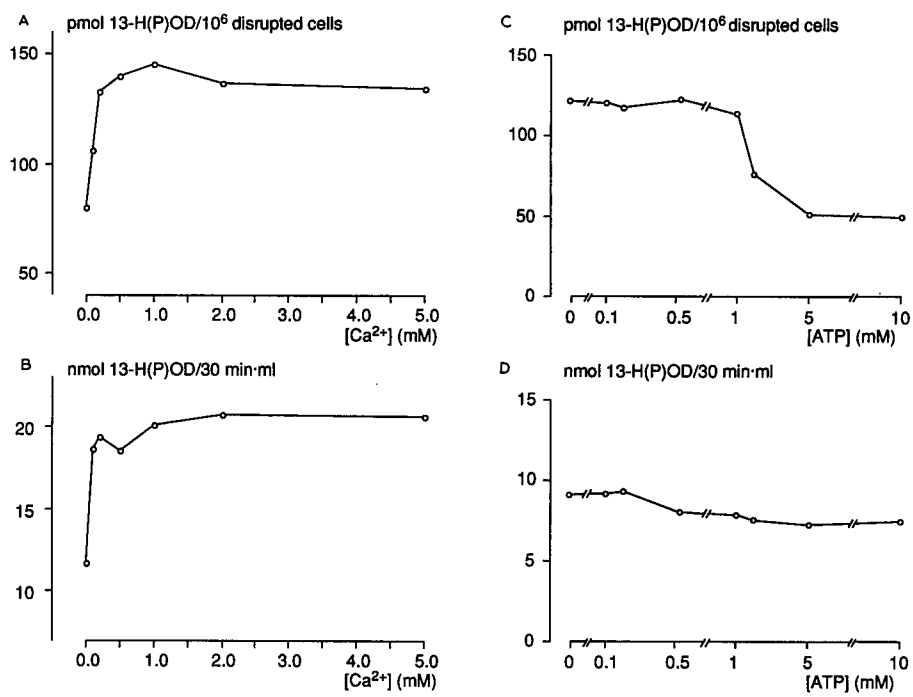

Fig. 2. Effect of added Ca^{2+} to 20000 × *g* supernatant of RBL-1 cells (A) or partially purified 12-lipoxygenase (B) when incubated with 60 μM linoleic acid for 30 min at 10 °C. Circles represent mean values of duplicate experiments with one enzyme preparation. Effect of added ATP to 20000 × *g* supernatant of RBL-1 cells (C) or partially purified 12-lipoxygenase (D) when incubated with 60 μM linoleic acid for 30 min at 10 °C. Circles represent values of duplicate experiments with one enzyme preparation.

appears that Ca^{2+} is nonessential for 12-lipoxygenase activity. Similar results were obtained by Hamasaki and Tai [3], who used arachidonic acid as substrate and observed an almost 3–5-fold stimulation in the presence of 0.5–5.0 mM Ca^{2+}. However, since 12-lipoxygenase activity, observed without adding Ca^{2+}, might be induced by low levels of Ca^{2+} from endogenous sources, cell supernatant was incubated with linoleic acid at increasing concentrations of either EDTA or EGTA. We found that 12-lipoxygenase activity was not affected by the addition of either EDTA or EGTA (0.1 nM–10 mM). Moreover, also with partially purified 12-lipoxygenase, which should be Ca^{2+} free, addition of Ca^{2+} was found to be nonessential for 12-lipoxygenase activity (Fig. 2B). The observed stimulation of 12-lipoxygenase activity was in the same order of magnitude as was observed for cell supernatant.

The 2-fold stimulation of 12-lipoxygenase activity by 0.2–5.0 mM Ca^{2+} (Fig. 2B) was not affected by the simultaneous addition of ATP (Sigma). This is in sharp contrast to the effect on 5-lipoxygenase activity. In the presence of both Ca^{2+} and ATP, 5-lipoxygenase activity was reported to be increased about 300-fold compared to either Ca^{2+} or ATP alone [13]. We found that 12-lipoxygenase was not stimulated by ATP alone. Using cell supernatant, even a decrease in 12-lipoxygenase activity of 60% was observed upon incubation in the presence of 5–10 mM ATP. As 12-lipoxygenase activity observed in the absence of added ATP might be induced by low levels of ATP from endogenous sources, cell supernatant was treated with alkaline phosphatase from calf intestinal mucosa immobilized on beaded agarose (Sigma) at a concentration of 2 U/ml at pH 8.0 for 15 min at 20 °C, to remove possibly endogenous ATP. Alkaline phosphatase was removed by centrifugation (5 min, 200 × *g*, 4 °C). However, no change in 12-lipoxygenase activity was observed after this treatment. ATP also did not stimulate partially purified 12-lipoxygenase. At the same ATP concentrations a larger inhibition of 12-lipoxygenase activity was observed in cell supernatant than in partially purified enzyme preparations. It is reasonable to suggest that the inhibition of 12-lipoxygenase in cell supernatant may be caused by ATP-dependent proteolysis of the enzyme.

In conclusion we found that Ca^{2+} and ATP were not essential for 12-lipoxygenase activity, although addition of Ca^{2+} could stimulate the enzyme activity about 2-fold. Moreover, 12-lipoxygenase in cell supernatant was not inactivated by preincubation at 1 mM Ca^{2+}; addition of EDTA prior to cell lysis did not improve recovery of 12-lipoxygenase. This in sharp contrast to the behaviour of 5-lipoxygenase, which was shown to be dependent on both Ca^{2+} and ATP for optimal activity. Addition of EDTA prior to cell lysis improved recovery of 5-lipoxygenase activity and in cell supernatant 5-lipoxygenase was rapidly inactivated by preincubation at 1 mM Ca^{2+}.

12-Lipoxygenases from various other sources were reported to be independent of Ca^{2+}, e.g, 12-lipoxygenases from rat platelets [3], bovine platelets, [5,15], bovine leukocytes [15], and porcine leukocytes [7]. So far no activation of human platelet 12-lipoxygenase by Ca^{2+} has been reported either. Only 12-lipoxygenase from human uterine cervix was reported to be stimulated about 2-fold in the presence of 1 mM Ca^{2+} and 2 mM ATP [16], but it remained unclear whether this stimulation would occur also in the presence of 1 mM Ca^{2+} alone, like we observed for 12-lipoxygenase from RBL-1 cells.

In addition to well known physiological functions of 13-HOD and 12-HETE [18–20] it was recently reported that 12-HETE may also be involved in the induction of heat shock proteins in human leukocytes [21], once more emphasizing the biological importance of 12-lipoxygenase.

This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (NWO). The authors wish to thank Drs. Josée Vervaart for skilful technical assistance.

References

1 Jakschik, B.A., Sun, F.F., Lee, L-H. and Steinhoff, M.M. (1980) Biochem. Biophys. Res. Commun. 95, 103–110.
2 Jakschik, B.A. and Kuo, C.G. (1983) Prostaglandins 25, 767–782.
3 Hamasaki, Y. and Tai, H-H. (1984) Biochim. Biophys. Acta 793, 393–398.
4 Van der Donk, E.M.M., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1991) Biochim. Biophys. Acta 1081, 135–140.
5 Walstra, P., Verhagen, J., Vermeer, M.A., Veldink, G.A. and Vliegenthart, J.F.G. (1987) Biochim. Biophys. Acta 921, 312–319.
6 Claeys, M., Kivits, G.A.A., Christ-Hazelhof, E. and Nugteren, D.H. (1985) Biochim. Biophys. Acta 837, 35–51.
7 Yokoyama, C., Shinjo, F., Yoshimoto, T., Yamamoto, S., Oates, J. and Brash, A.R. (1986) J. Biol. Chem. 261, 16714–16721.
8 Nugteren, D.H. (1975) Biochim. Biophys. Acta 380, 299–307.
9 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
10 Van Os, C.P.A., Vente, M. and Vliegenthart, J.F.G. (1979) Biochim. Biophys. Acta 574, 103–111.
11 Verhagen, J., Veldink, G.A., Egmond, M.R., Vliegenthart, J.F.G. Boldingh, J. and Van der Star, J. (1978) Biochim. Biophys. Acta 198, 449–459.
12 Goetze, A.M., Fayer, L., Bouska, J., Bornemeier, D. and Carter, G.W. (1985) Prostaglandins 29, 689–701.
13 Hogaboom, G.K., Cook, M., Newton, J.F., Varrichio, A., Shorr, R.G.L., Sarau, H.M. and Crooke, S.T. (1986) Molec. Pharmacol. 30, 510–519.
14 Haurand, M. and Flohé, L. (1988) Biol. Chem. Hoppe-Seyler 269, 133–142.
15 Takahashi, Y., Ueda, N. and Yamamoto, S. (1988) Arch. Biochem. Biophys. 266, 613–621
16 Flatman, S., Hurst, J.S., McDonald-Gibson, R.G., Jonas, G.E.G. and Slater, T.F. (1986) Biochim. Biophys. Acta 883, 7–14.

17 Wong, A., Hwang, S.M. Cook, M.N., Hoogaboom, G.K. and Crooke, S.T. (1988) Biochemistry 27, 6763–6769.
18 A.A. Spector, Gordon, J.A. and Moore, S.A. (1988) Prog. Lipid Res. 27, 271–323.
19 Haas, T.A., Bastida, E., Nakamura, K., Hullin, F., Admirall, L. and Buchanan, M.R. (1988) Biochim. Biophys. Acta 961, 153–159.
20 Nugteren, D.H. and Kivits, G.A.A. (1987) Biochim. Biophys. Acta 921, 135–141.
21 Köller, M. and König, W. (1991) Biochem. Biophys. Res. Commun. 175, 804–809.