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Evidence for a leukotriene A_4 hydrolase in *Xenopus laevis* skin exudate

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Abstract Leukotriene A_4 hydrolase is a cytosolic metalloenzyme of the arachidonic acid biosynthetic pathway responsible for leukotriene A_4 conversion into leukotriene B_4. In addition to its epoxide hydrolase properties, this enzyme exhibits an aminopeptidase activity which was used as an assay to monitor the purification of a novel form of leukotriene A_4 hydrolase from *Xenopus laevis* skin exudate. This 70 kDa, secreted, form of leukotriene A_4 hydrolase was identified by immunochemical cross-reactivity with anti-human leukotriene A_4 hydrolase antibodies and by its capacity to convert leukotriene A_4 into leukotriene B_4. Moreover this enzyme produced a second metabolite which could be the leukotriene B_4 isomer 5S,12R-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid, previously shown by Strömberg et al. (Eur. J. Biochem. 238 (1996) 599–605) to be formed by incubation of the leukotriene A_4 with amphibian tissue extracts. Partial amino acid sequencing of peptides generated by endolysin C fragmentation of the purified enzyme confirmed the presence, in *X. laevis* skin secretions, of a related but distinct form of leukotriene A_4 hydrolase which is likely to be responsible for the production of these eicosanoid metabolites of leukotriene A_4.

Key words: Aminopeptidase; Leukotriene A_4 hydrolase; Leukotriene B_4; Dactylysin; Eicosanoid; (*Xenopus laevis*)

1. Introduction

Leukotrienes belong to a family of eicosanoids derived from arachidonic acid which biological activity is related to the inflammatory response [1]. The biosynthetic pathway leading to leukotriene A_4 (5S-trans-5,6-oxido-7,9-trans-11,14-cis-eicosatetranoic acid) involves conversion of arachidonic acid by the 5-lipoxygenase into a labile epoxide, leukotriene A_4 (LTA_4) which is converted by leukotriene A_4 hydrolase into leukotriene B_4 (LTB_4: 5S,12R-dihydroxy-6,14-cis,8,10-trans-eicosatetranoic acid).

Leukotriene A_4 hydrolase is considered as a soluble, cytosolic enzyme, which behaves as a 69 kDa monomer. It was purified and characterized starting from various cell types and organisms including guinea pig lung [2] and liver [3], human lung [4,5], human neutrophils [6] human erythrocytes [7], rat neutrophils [8] and the Raji, B-lymphocyte cell line [9]. Furthermore, the cDNA clone coding for LTA_4 hydrolase was isolated from human placenta [10], human spleen [11] and rat mesangial cells [12]. The human LTA_4 hydrolase cDNA was expressed in *Escherichia coli* [13] and in cultured *Spodoptera frugiperda* insect cells [14]. So far LTA_4 hydrolase has been recognized as a Zn^{2+}-metalloenzyme [15] and as a bifunctional hydrolase with intrinsic aminopeptidase activity [16]. Moreover it was recently demonstrated that the amino acid sequence of aminopeptidase-B (Ap-B) from the rat testis exhibits 33% identity and 48% similarity with leukotriene A_4 hydrolase, a relation further supported by the capacity of Ap-B to hydrolyze in vitro leukotriene A_4 [17].

In the course of purifying dactylysin (EC 3.4.24.60) from *Xenopus laevis* skin secretions, a Zn^{2+}-metalloendopeptidase with a thermolysin-like selectivity for bulky hydrophobic amino acid side chains [18,19], we have copurified a protein entity detected and monitored by mean of its apparent aminopeptidase B-like activity. In the present paper we show that this enzyme exhibits in vitro biochemical properties of a leukotriene A_4 hydrolase and is structurally related to that enzyme.

2. Materials and methods

2.1. Materials

X. laevis were obtained from the Centre National de la Recherche Scientifique, Montpellier, France. Epinephrine, bovine serum albumin, PMSF, GEMSA, bestatin, iodacetamide, orthophenanthroline, and prostaglandin B_1 were purchased from Sigma. Leukotriene A_4 methyl ester, leukotriene B_4, 6-trans-leukotriene B_4 and 6-trans-12-epi-leukotriene B_4 were from Cayman Chemical (Ann Arbor, MI, USA). Peptides [Arg^0,Leu^5,Arg^6] enkephalin and [Arg^0,Leu^5] enkephalin were synthesized in our laboratory by the solid-phase method [20] using a NPS 4000 semi-automated multisynthesizer (Neosystem, France). Peptide purification and analysis were routinely conducted using a set of analytical techniques including HPLC, amino acid composition, N-terminal sequencing and fast atomic bombardment mass spectrometry as in [21]. The peptide Arg-Tyr-Gly-Gly-Phe and Leu^5 enkephalin were obtained from Eurogentec (Belgium) and Neosystem respectively.

2.2. X. laevis skin exudate preparation and enzyme purification procedure

Exudate was obtained after dorsal injection of epinephrine (100–200 μg per animal) and collected by scrapping the frog skin. It was diluted with about 5 volumes of Tris-HCl 50 mM pH 7.5 and dialyzed exhaustively against the same buffer for 48 h. The dialyzed material was subjected to ion exchange chromatography on DEAE Tris-acryl M (IBF, Villeneuve la Garenne, France) and eluted with 50 mM Tris-HCl, pH 7.5 buffer. This allowed to eliminate more than 90% of the exudate proteins. The active fractions were recovered in the void volume, pooled and concentrated to a 15 ml final volume by using ultra-free units (Millipore, Saint-Quentin Yvelines, France). Three ml of the enzymatically active sample was subjected to molecular sieve filtration on a Sephacryl S-100 column (280 ml) equilibrated with 50 mM Tris-HCl, pH 7.5 buffer. The active fractions were pooled and concentrated to be submitted to another molecular sieve filtration on a Sephadex G-150 (90 ml) column equilibrated with the same buffer. Under such conditions the recovered enzyme activity was found electrophoretically homogeneous on SDS-PAGE. An alternative strategy used a chromatofocusing step after the DEAE Tris-acryl ion exchange separation (see above). Polybuffer exchanger 94 (PBE 94, Pharmacia) with a pH range 5 to 8 was used. The column (20 ml of PBE 94) was first equilibrated in 0.025 M Tris-acetic acid pH 8.3. Five ml of the concentrated active fractions recovered from the DEAE Tris-acryl step were applied to the column and were eluted with a mixture of polybuffer 96 and 74 (30/70; v/v), adjusted to pH 5.0 with acetic acid to form a pH gradient ranging from 5 to 8. 75 fractions (2 ml) were

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recovered, separately concentrated (final volume 200 μl) then rinsed with the Tris-HCl buffer (50 mM, pH 7.5) using ultra-free units to eliminate ampholytes and to assay the enzyme activity. All columns were run at 4°C. The partially purified enzyme was stable for several months at 4°C.

2.3. Assay of aminopeptidase activity

Aminopeptidase activity: the enzyme assays were carried out by incubating 5 μg of either substrates; i.e. [Arg^0,Leu^5,Arg^6] enkephalin, [Arg^0,Leu^5] enkephalin, Arg-Tyr-Gly-Gly-Phe, [Leu^5,Arg^6] enkephalin or [Leu^5] enkephalin with an aliquot of the enzyme preparation in a 25 μl final volume of 50 mM Tris-HCl buffer (pH 7.5) during 3 h at 37°C. The reaction mixture was then applied onto the top of an RP-HPLC column (Kromasyl C18, 5 μm, 100 Å; 250×4.6 mm from AIT, Saint Nom la Bretèche, France) and eluted with a linear gradient (10 to 45% acetonitrile in 0.05% TFA) over 18 mn (flow rate: 1 ml mn^{-1}). The resulting peptide fragments were monitored by UV absorbance at 215 nm and were identified both by their retention times by reference to standards and by their amino acid composition. These were performed according to the HPLC Amino Acid Analysis System Pico-Tag (Waters chromatography division, Milford, MA).

2.4. Effects of inhibitors

Aliquots of the enzyme preparation were preincubated with various inhibitors at the shown concentrations (see Section 3) for 15 min at 37°C in Tris-HCl buffer pH 7.5. The reaction was initiated by addition of 5 μg [Arg^0,Leu^5,Arg^6] enkephalin in a 25 μl final volume and carried out for 3 h at 37°C. Results were compared to those obtained without inhibitors.

2.5. Assay of leukotriene A_4 hydrolase activity

LTA_4 methyl ester was first converted into the free acid by alkaline hydrolysis according to the manufacturer's instructions (Cayman Chemical). Aliquots (30 μl) of active enzyme preparation were incubated at room temperature with 25 or 50 μm LTA_4 (final volume: 40 μl). After 30 s, the reaction was stopped by addition of 1 volume of methanol. The samples were acidified with acetic acid (5 μl, 0.1 M) and were analyzed by reverse-phase HPLC (Kromasyl column C18) using an isocratic mixture of acetonitrile/methanol/water/acetic acid (25/40/35/0.01; v/v/v/v). Prostaglandin B1 (PGB1) was used as an internal standard. The absorbance was monitored at 270 nm.

2.6. Gel electrophoresis

Gel electrophoretic analysis was performed on aliquots of concentrated active enzyme fractions under denaturing conditions on 7.5% or 10–15% acrylamide gels using the Phast System (Pharmacia, Uppsala, Sweden). Proteins were revealed by silver staining.

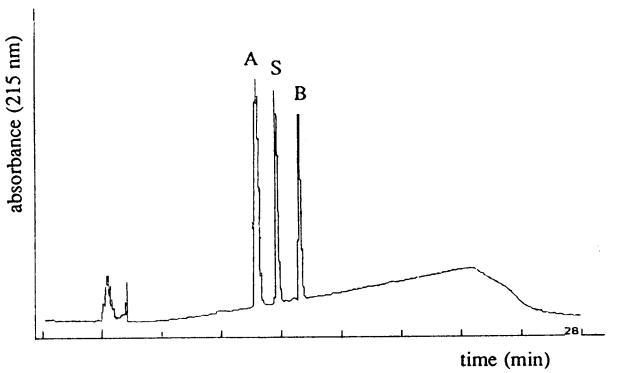

Fig. 1. HPLC analysis of peptide fragments generated by exposure of [Arg^0,leu^5,Arg^6] enkephalin (S) to the enzyme active fractions containing both aminopeptidase and dactylysin activities and recovered at various stages of the purification procedure (after DEAE Sephacryl and Sephacryl S-100 steps). After incubation of 5 μg (S) with an aliquot (20 μl) of enzyme active fraction for 3 h in Tris-HCl, 50 mM, pH 7.5 at 37°C, the reaction mixture was applied onto the top of the HPLC column and was eluted with a gradient as described in Section 2. A and B were identified as peptides Arg-Tyr-Gly-Gly-Phe and Tyr-Gly-Gly-Phe-Leu-Arg respectively. The pattern here shown is an example of the routine analysis on incompletely separated form of the two enzyme activities.

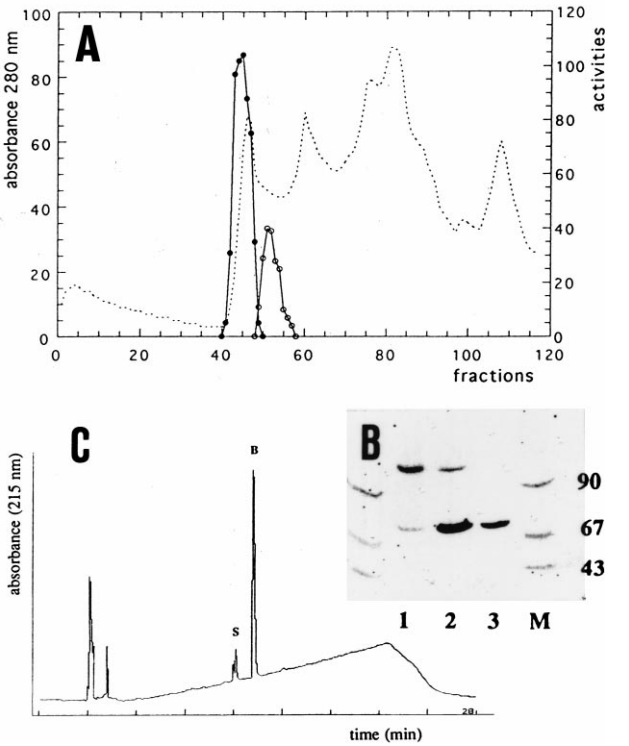

Fig. 2. A: Separation of datylysin (○) and aminopeptidase B-like (●) activities by molecular sieve filtration on a Sephadex G-150 column in Tris-HCl, 50 mM, pH 7.5. The activities expressed as arbitrary units were monitored on 20 μl aliquots of each 1 ml recovered fraction as described in Section 2 using (S: Arg-Tyr-Gly-Gly-Phe-Leu-Arg) as substrate (see legend to Fig. 1). A_{280nm} (...). B: SDS-PAGE electrophoresis on the Phast System on polyacrylamide 7.5% gels. Each of the analyzed 1 ml fraction was concentrated to about 50 μl final volume and a 4 μl aliquot was submitted to electrophoresis. Lanes 1, 2 and 3 correspond to fractions 42, 44 and 46 respectively from molecular sieve filtration of A. Lane M: molecular mass markers in kDa. C: HPLC test of aminopeptidase B-like activity (arbitrary units) on fraction 46 (see A and Fig. 3A, lane 3). Details in Section 2 and legend to Fig. 1. S, Arg-Tyr-Gly-Gly-Phe-Leu-Arg; B, Tyr-Gly-Gly-Phe-Leu-Arg.

2.7. Western blot analysis

Protein samples were submitted to 8% SDS-PAGE. Electrotransfer of proteins to a nitrocellulose membrane (0.45 μm, Schleicher and Schuell, Dassel, Germany) was performed with a semi-dry blotting apparatus (Hoefer). LTA_4 hydrolase was detected using polyclonal anti-human LTA_4 hydrolase antibodies (Cayman Chemical; 1/2000 dilution) as described in [17,22].

3. Results

X. laevis skin exudate was used as a source of dactylysin, a metalloendopeptidase which activity was monitored by HPLC analysis of peptide fragments generated by selective cleavage at the Phe-Leu bond of [Arg^0,Leu^5,Arg^6] enkephalin, an heptapeptide best recognized by the enzyme [18]. At each step of dactylysin purification it was observed that beside peptide A (Arg^0-Tyr-Gly-Gly-Phe; Fig. 1) i.e. the main product resulting of dactylysin action on the heptapeptide substrate (S), a second product, called peptide B, was formed (Fig. 1). This was identified both by its amino acid composition and by its com-

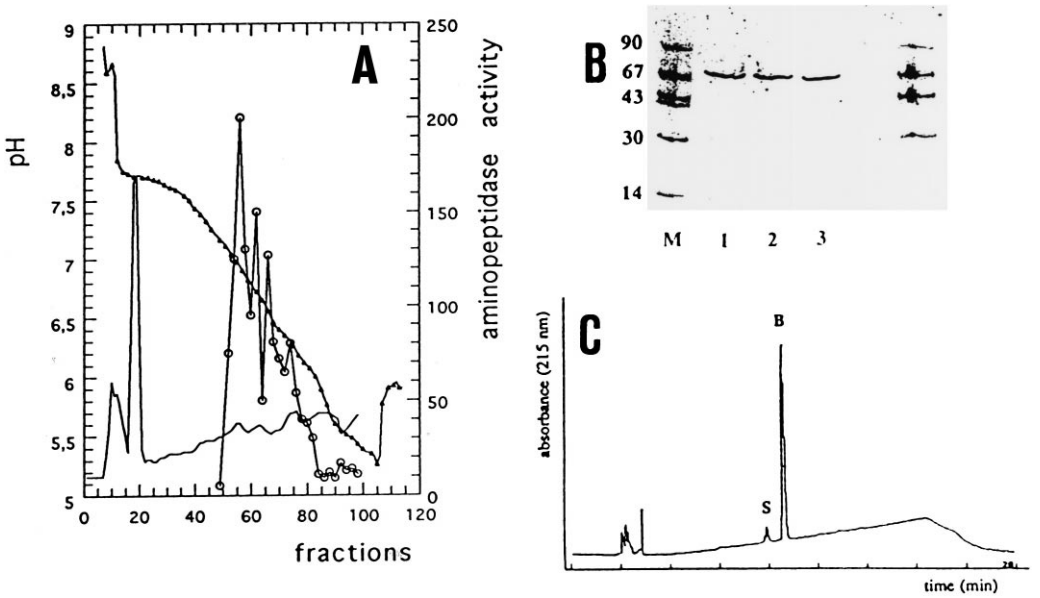

Fig. 3. A: Chromactofocusing analysis of aminopeptidase B-like activity. Elution profile observed by measurement of aminopeptidase B-like activity (○: arbitrary units) as described in details in Section 2 on 2 ml fractions. A_{280nm} (—) and pH (▲). B: SDS-PAGE (10–15%) on the Phast System. Lanes 1, 2 and 3 represent 4 μl aliquots of previously concentrated fractions 55 (220 μl), 56 (215 μl) and 57 (165 μl) respectively. Lane M: molecular mass markers in kDa. C: HPLC test of aminopeptidase B-like activity (arbitrary units) on fraction 56 (see A and B, lane 2) Details in Section 2 and Fig. 1. S, Arg-Tyr-Gly-Gly-Phe-Leu-Arg; B, Tyr-Gly-Gly-Phe-Leu-Arg.

parison with standard peptides, as representing Tyr-Gly-Gly-Phe-Leu-Arg and was assumed to result from the action of an aminopeptidase B-like activity on the peptide substrate. Indeed both dactylysin and aminopeptidase activities were not separated at the DEAE-Sephadex step and were coeluted together with the unadsorbed proteins (not shown); the latter represented roughly 10% of the total applied onto the top of the column. Whereas the two activities could not be entirely separated by exclusion chromatography on Sephacryl S-100 (not shown), molecular sieve filtration on Sephadex G-150 clearly achieved a significant separation (Fig. 2A). After pooling and concentration of the relevant G-150 fractions containing the aminopeptidase activity, SDS-PAGE analysis of the corresponding protein indicated that this enzyme reaction was performed by a 70 kDa protein entity (Fig. 2B, lane 3 and Fig. 2C).

Purification of the aminopeptidase activity by chromatofocusing revealed a microheterogeneity with species of respective apparent p*I* 6.3, 6.5, 6.7, 6.9 (Fig. 3A). The latter was found to be free of endopeptidase activity and of any contaminant

Table 1
Representation of major (▼), and minor (▾ and ↓) cleavages produced by aminopeptidase B-like activity of *X. laevis* skin secretion LTA_4 hydrolase in a series of enkephalin-related peptides

Arg▼Tyr-Gly-Gly-Phe-Leu Arg
Arg▼Tyr-Gly-Gly-Phe-Leu
Arg↓Tyr-Gly-Gly-Phe
Tyr↓Gly-Gly-Phe-Leu-Arg
Tyr↓Gly-Gly-Phe-Leu

on SDS-PAGE using the Phast System (70 kDa band on Fig. 3B, C). This 70 kDa species exhibited, together with an aminopeptidase B-like activity toward [Arg^0,Leu^5] enkephalin and Arg-Tyr-Gly-Gly-Phe which were transformed in [Leu^5] enkephalin and Tyr-Gly-Gly-Phe respectively, a weaker but broader aminopeptidase activity. This was shown on such peptides as [Leu^5,Arg^6] enkephalin and [Leu^5] enkephalin, both substrates from which the N-terminal tyrosine residue was eliminated (summarized in Table 1) by leukotriene A_4 hydrolase as previously described by Griffin et al. [23] and Nissen et al. [24].

The enzyme was not inhibited by either PMSF (1 mM), GEMSA (250 μM), and iodacetamide (1 mM); it is neither serine nor thiol protease. Its activity was abolished by either bestatin (0.2 mM) or orthophenanthroline (1 mM) confirming both its amino- and metallopeptidase character.

Western blot analysis indicated that whereas the 70 kDa species was not recognized by the aminopeptidase-B antibodies (not shown), a strong reaction was obtained by using anti-human LTA_4 hydrolase antibodies (Fig. 4).

Final demonstration that the 70 kDa species with exopeptidase activity was indeed LTA_4 hydrolase was achieved:

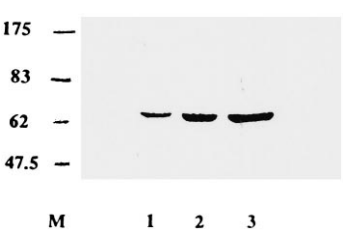

Fig. 4. Western blot analysis of aminopeptidase B-like activity. The enzyme active fractions recovered after Sephacryl S-100 chromatography (see Section 2) were run on 8% SDS-PAGE and analyzed by immunochemical blotting using polyclonal anti-human LTA_4 hydrolase antibodies (1/2000 dilution). Lanes 1, 2 and 3 represent same sample at three different concentrations (1, 3 and 7).

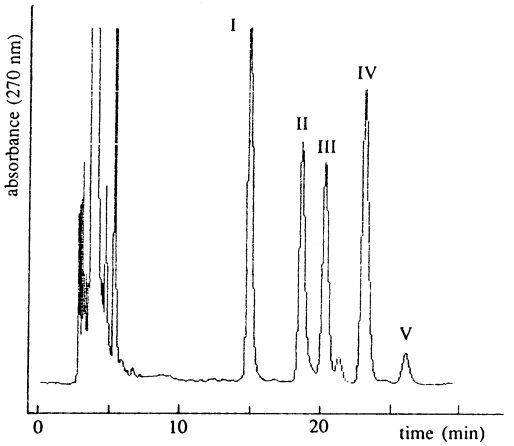

Fig. 5. HPLC analysis of the products generated by the action of purified *X. laevis* skin secretion LTA_4 hydrolase on LTA_4. Details of the procedure in Section 2. A_{270nm} (arbitrary units). Peaks I: prostaglandin B1; II and III: non-enzyme degradation products of LTA_4; IV: LTB_4; V: compound X (see text for details).

(i) by showing its capacity to convert LTA_4 into LTB_4 (Fig. 5) and (ii) by determining the amino acid sequence of three peptides obtained by endolysin C fragmentation of the pure protein. The microsequencing of octapeptide, hexapeptide and undecapeptide fragments indicated respectively 87.5%, 100% and 36% sequence similarity and respectively 62.5%, 100% and 21% sequence identity with the corresponding reference peptides from human LTA_4 hydrolase sequence (not shown; [10]). Noticeably the LTA_4 hydrolase activity was clearly detected at all stages of purification, i.e. after the DEAE Sephacryl, Sephacryl S-100 and Sephadex G-150 chromatography and chromatofocusing steps (not shown). Finally a secondary product was observed in the HPLC analysis of the products of LTA_4 conversion into LTB_4 (marked V on Fig. 5). In the different studied extracts, this compound appeared in a fairly constant ratio relative to LTB_4 (not shown) as observed by Strömberg et al. [25]. This observation strongly suggested that this could represent compound X (5S,12-dihydroxy-6,10-trans-8,14-cis-eicosatetranoic acid) previously detected [25] in frog tissue extracts.

4. Discussion

Previous work from [25] has suggested the presence of LTA_4 hydrolase in *X. laevis* on the basis of the detection of LTB_4 in the nine amphibian organs tested. Additionally incubations of supernatants of these organ homogenates with LTA_4 revealed the formation of the unexpected compound X which was shown to be 5S,12R-dihydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid. The authors concluded that this compound was resulting from an activity associated, indirectly or directly, to leukotriene A_4 hydrolase.

In the present report we have demonstrated unequivocally that *X. laevis* skin exudate contains an enzyme activity capable to hydrolyze LTA_4 into LTB_4 and also to convert part of substrate into a compound, probably corresponding to component X described by Strömberg et al.; both products were formed in vitro by the same purified LTA_4 hydrolase.

Site-directed mutagenesis observations on human recombinant LTA_4 hydrolase at a key residue of the catalytic site, i.e. mutations Tyr 378 F and Tyr 378 Q, both generated a mutated form of the enzyme capable to produce in vitro compound X [26]. Partial sequencing of endolysin C peptide fragments of the presently described LTA_4 hydrolase has shown a high degree of sequence similarity in two domains (87.5 and 100%) but clear discrepancies on another peptide of the human LTA_4 hydrolase reference enzyme. These observations suggest that the *X. laevis* LTA_4 hydrolase might be a closely related enzyme but possessing selective amino acid modifications which could account for the capacity of this amphibian enzyme to produce the component X. Determination of the complete sequence of the cloned enzyme cDNA should clarify this issue and help establishing the molecular basis for this catalytic difference and particularly verify whether the protein has an intrinsic mutation in the Tyr 378 position. Interestingly Tyr 378 is replaced by a phenylalanine in the primary sequence of a protein from *Dictyostelium discoideum*, presumed to be a LTA_4 hydrolase (accession number: V27538; Iho and Kopachik, 1995, unpublished data; [17]).

Another interesting point deals with the presence of *X. Laevis* LTA_4 hydrolase in skin secretions. Although exudation process differs from classical exocytose since the secretory granules conserved their membranes after expulsion, it could be hypothesized that the LTA_4 hydrolase described in this paper possesses structural properties allowing for its secretion. Noticeably although LTA_4 hydrolase was established as a cytosolic enzyme, its presence was reported in human plasma extracts [27].

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