Protein Expression and Purification 60 (2008) 1–6

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

High-level expression, purification, and crystallization of recombinant rat leukotriene C_4 synthase from the yeast *Pichia pastoris*

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ARTICLE INFO

Article history:
Received 17 November 2007
and in revised form 19 February 2008
Available online 23 March 2008

Keywords:
LTC4S
Crystallization
Detergent screen
Membrane protein
Pichia pastoris

ABSTRACT

Leukotriene C_4 synthase (LTC4S) is a member of the MAPEG family of integral membrane proteins and catalyzes the conjugation of leukotriene A_4 with glutathione to form leukotriene C_4, a powerful mediator of allergic inflammation and anaphylaxis. Structural information on this class of proteins would be highly useful for rational drug design. Here, we report the expression, purification, and crystallization of recombinant LTC4S from rat. The enzyme was expressed as an N-terminal hexa-histidine-tagged fusion protein in *Pichia pastoris* and purified with two steps of affinity chromatography on Ni–Sepharose and *S*-hexylglutathione agarose, followed by gel filtration. From 1 l culture, we obtained 0.5–1 mg of apparently homogeneous protein with a specific LTC4S activity ranging between 36 and 49 μmol/mg/min. A small-scale screen identified dodecyl maltoside as a useful detergent for protein extraction and yielded a highly active protein. When tested separately in crystallization trials of the purified LTC4S, six out of seven detergents from all the maltoside family yielded diffracting crystals with the highest resolution at ~6 Å. Hence, our approach holds promise for solving the structure of rat LTC4S and other members of the MAPEG family of integral membrane proteins.

Leukotriene (LT)[3] C_4, D_4, and E_4 are conjugates of glutathione and the arachidonic acid derivative leukotriene A_4 (LTA_4). These conjugates are collectively known as cysteinyl-leukotrienes (cys-LTs) and are powerful lipid mediators of allergy and anaphylaxis [1]. In the airways they induce bronchoconstriction, mucus secretion, and edema formation, which are the cardinal signs of asthma. Accordingly, experimental and clinical data clearly demonstrate the importance of cys-LTs in the pathophysiology of asthma [2], and recently, specific drugs targeted against these mediators were marketed as new medications against this disease [3].

Leukotriene C_4 synthase (LTC4S) is an integral membrane enzyme with a molecular mass of 18 kDa [4–6] located in the outer nuclear membrane and peripheral endoplasmic reticulum [7]. When the enzyme was cloned and sequenced, a previously unknown homology with 5-lipoxygenase activating protein (FLAP) was demonstrated [8,9]. Further work identified two microsomal GSH transferases (MGST2 and MGST3), which both possess LTC_4 synthase activity and exhibit a high degree of similarity to both LTC4S and FLAP [10,11]. These proteins, together with the detoxifying enzyme MGST1 and the microsomal PGE-synthase type 1 (mPGES1), are members of the MAPEG family (membrane associated proteins in eicosanoid and glutathione metabolism) [12].

Due to the technical hurdles associated with membrane protein crystallography, structural information on human integral membrane proteins is scarce. Access to large amounts of pure and solubilized membrane protein, as well as identification of the optimal detergent(s) for extraction and crystallization, are key issues for success [13–15]. Thus, we recently reported a rapid and cost-efficient strategy to screen for extracting detergents on recombinant membrane proteins expressed in *Escherichia coli* (*E. coli*) [13,16]. However, the usefulness of this method has not yet been tested on other expression systems.

Here, we report the purification, detergent screening, and crystallization of recombinant rat LTC4S which was overexpressed at

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[3] *Abbreviations used:* LT, leukotriene; GSH, glutathione; cys-LT, cysteinyl-leukotriene; 5-LO, 5-lipoxygenase; LTA_4, 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTC_4, 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTD_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTE_4, 5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTC4S, LTC_4 synthase; MGST, microsomal GSH *S*-transferase; MAPEG, membrane associated proteins in eicosanoid and glutathione metabolism; mPGES1, microsomal PGE-synthase 1; IMAC, immobilized metal affinity chromatography.*Enzymes:* Leukotriene C_4 synthase (EC 2.5.1.37); glutathione *S*-transferase (EC 2.5.1.18) (not specific for microsomal GSH *S*-transferase type 2 or type 3).

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doi:10.1016/j.pep.2008.03.005

high levels in the yeast *Pichia pastoris (P. pastoris)*. The protocol yields large amounts of solubilized and enzymatically active protein in maltoside detergents allowing growth of crystals which initially diffract to ~6 Å resolution. Hence, we believe this is a useful approach for solving the structure of rat LTC4S and other members of the MAPEG family of enzymes and proteins, as it was highly useful for solving the structure of human LTC4S [17].

Materials and methods

Materials

All chemicals were of analytical grade and, unless otherwise stated, obtained from Sigma. All detergents were purchased from Anatrace Inc. LTA_4 methyl ester (Biomol) was saponified with LiOH (6% v/v) in tetrahydrofuran for 48 h at 4 °C to produce LTA_4. LTC_4 and PGB_2 were purchased from Biomol. Yeast Nitrogen Base was obtained from Invitrogen.

Protein expression

Rat LTC4S cDNA [18] with an N-terminal sequence encoding a $(His)_6$-tag was subcloned into the expression vector pPICZA (Invitrogen) using the following set of primers; Forward primer with an EcoR1 site and the (His)6-tag, 5′-GCG CGA ATT CAT AAT GTC TCA CCA TCA TCA CCA CCA TAA GGA AGA AAC; Reverse primer with a Not1 site, 5′-GAG AGA GCG GCC GCT CAG GCC ATC GGC AGG AGC. The protein coding part of the resulting plasmid was verified by DNA sequencing and transformed into *P. pastoris* KM71H cells using the Pichia EasyComp Transformation kit (Invitrogen). Recombinant cells were cultivated in baffled flasks in 2.5 L minimal yeast medium with glycerol at 27 °C. When OD_{600} reached 8–10, the cells were resuspended in 0.5 L minimal yeast medium with 0.5% methanol. The cells were harvested after 72 h by centrifugation (2500*g*, 7 min) and resuspended in 25 mM phosphate buffer, pH 7.8, 100 mM KCl, and 10% glycerol.

Homogenization was performed with glass beads (0.5 mm) using a Bead beater (BioSpec Products, Inc.) operated 7 × 1 min on ice. The slurry was filtered through nylon net filters (180 μm, Millipore) and centrifuged (1500*g*, 10 min).

Detergent screen

The detergent screen was performed as previously described [13,16] with some modifications. Aliquots of 100 μl from the 1500*g* supernatant were divided in a 96-well plate and mixed with detergents according to Table 2. Solubilization was performed by shaking at 900 rpm at +4 °C for 30 min. The solubilized material was filtered through a 96-well filter plate (0.65 μm, Millipore) and the filtrates were mixed with 25 μl Ni–NTA agarose (Invitrogen). After 10 min incubation at +4 °C, the unbound material was passed through a similar filter plate by centrifugation at 100*g* for 1 min. The resins were then washed three times with 200 μl binding buffer (20 mM Na-phosphate, 300 mM NaCl, 40 mM imidazole, and 0.5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), pH 7.5) containing the appropriate detergent (referred to as the second detergent, Table 2). The histidine-tagged protein was eluted with 400 mM imidazole in the binding buffer with the same detergent. The purity and the yield of the protein were judged by SDS–PAGE stained with Coomassie Brilliant Blue.

Table 2
Detergents screened for extraction and purification of rat LTC4S

	Extracting detergent (w/v)	Second detergent (w/v)
Triton X-100/sodium deoxycholate (TX100DOC)	1%[a]/0.5%	–
CHAPS	2%	–
LDAO	1%	–
HEGA-10	1%	–
Cymal-5	1%	0.25%
Dodecyl maltoside	1%	0.03%
Fos-choline-12	1%	–
Octyl glucoside	2%	1%
Cymal-6	–	0.1%
Cymal-7	–	0.03%
Decyl maltoside	–	0.3%
Undecyl maltoside	–	0.1%
Nonyl glucoside	–	0.6%

[a] The concentration percentage is v/v.

Protein purification

The overall course of purification is summarized in Table 1. Membrane bound proteins in the 1500*g* supernatant were solubilized with Triton X-100 (1%, v/v) and sodium deoxycholate (0.5%, w/v) (TX100DOC) for 1 h with stirring on ice. After centrifugation (10,000*g*, 10 min) the supernatant was supplemented with 10 mM imidazole and loaded on a column packed with 5 ml Ni–Sepharose Fast Flow (GE Healthcare Biosciences) by gravity flow. The column was washed with three column volumes of buffer A (25 mM phosphate buffer, pH 7.8, 10% glycerol, 0.1% Triton X-100, and 5 mM 2-mercaptoethanol) supplemented with 20 mM imidazole and 0.1 M NaCl, followed by an equal volume of buffer A containing 40 mM imidazole and 0.5 M NaCl. LTC4S was eluted with buffer A supplemented with 300 mM imidazole and 0.5 M NaCl. The next purification step was performed on a column packed with 3 ml *S*-hexyl-glutathione agarose. The column was washed with 3 column volumes of buffer A supplemented with 0.5 M NaCl. Pure LTC4S was eluted with four column volumes of 25 mM phosphate buffer, pH 7.8, 0.1% Triton X-100, 30 mM probenecid, and 5 mM 2-mercaptoethanol. The sample was desalted on PD-10 columns (GE Healthcare Biosciences) and eluted in 25 mM phosphate buffer, 10% glycerol, 0.05% Triton X-100, and 5 mM 2-mercaptoethanol. After a fivefold concentration on an Amicon Ultra-15 centrifugal filter device with a 10 kDa cut-off (Millipore) the protein was stored frozen at −20 °C.

Table 1
LTC4S from 4 l cell culture was purified as described in the Methods section, Aliqots were removed after indicated purification steps and incubated with LTA_4 (35 μM) for 15 s at room temperature

	Volume (ml)	Protein conc (mg/ml)	Amount protein (mg)	Activity (μmol/mg/ml)	Total activity (μmol)	Specific activity (μmol/mg/min)	Purification (-fold)	Yield (%)
Homogenate	220	1,7	374	0,49	108	0,29	1	100
Solubilized	226	1,74	393	1,39	314	0,80	2,76	291
IMAC	30	0,13	3,9	2,54	76	19,5	67	70,4
S-Hexyl-glutathione	19	0,14	2,9	1,34	29	9,6	33	26,8
After desalt and concentration	1	1,14	1,1	32	35	28	97	32,6

Formation of LTC_4 was analyzed by RP-HPLC.