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Recombinant Laforin for Structural Studies

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1. Introduction

Lafora disease or Lafora progressive myoclonic epilepsy was first described in 1911 by Gonzalo Lafora (Lafora, 1911; Lafora & Glueck, 1911). The disease belongs phenomenologically to the progressive myoclonic epilepsies (Monaghan & Delanty, 2010) and is the most prevalent of such conditions, which include myoclonic epilepsy with ragged-red fibers, action myoclonus-renal failure syndrome, Kuf's disease, infantile neuroaxonal dystrophy, the sialidoses and Unverricht-Lundborg disease (Baltic myoclonus) (Monaghan & Delanty, 2010). These diseases afflict initially healthy children with ever-worsening and soon intractable myoclonus and epilepsy, usually associated with neurodegeneration, eventual dementia and early death (Ramachandran et al., 2009).

The first symptoms of Lafora disease appear between the ages of 8 and 18 years. There is an insidious, near-simultaneous or rapidly successive onset of headaches, difficulties in school work, myoclonic jerks, generalized seizures and in many cases, visual hallucinations of both epileptic and psychotic origin. The myoclonus, seizures and hallucinations gradually worsen and become intractable. For many years, the patient struggles to maintain normal contact and communication, interrupted by extremely frequent myoclonic absence seizures. Gradually, dementia sets in, and by 10 years after onset, the patient is in near continuous myoclonus with absences, frequent generalized seizures, and profound dementia or a vegetative state (Minassian, 2001; Ramachandran et al., 2009; Striano et al., 2008).

A distinctive pathology characterizes Lafora disease. Cells of various types exhibit dense accumulations of malformed and insoluble glycogen molecules, termed polyglucosans, which lack the symmetric branching that allows glycogen to be soluble. These polyglucosan accumulations are called Lafora bodies, and in the central nervous system, they are present profusely in all brain regions and in the majority of neurons, specifically in the cell bodies and dendrites (Minassian, 2001; Ramachandran et al., 2009; Striano et al., 2008).

Genetically, Lafora disease is an autosomal recessive disease caused by mutations in two genes: (1) the EPM2A gene, located on chromosome 6 at 6q24, coding for the protein laforin, a dual-specificity phosphatase (DSP), accounting for the largest group of patients (Minassian et al., 1998) and (2) the EPM2B (NHLRC1) gene, located on chromosome 6 at 6p22, coding for the protein malin, an E3 ubiquitin ligase that interacts with laforin (Chan et al., 2003a; Chan et al., 2003b). About 20% of cases are not explained by abnormalities in genes coding for laforin or malin (Singh & Ganesh, 2009); it is postulated that the mutations involved in

these cases involve either an unknown third gene or a regulatory sequence for the EPM2A or EPM2B genes (Monaghan & Delanty, 2010; Singh & Ganesh, 2009).

Laforin is the only human DSP known to have a carbohydrate binding module (CBM) responsible for targeting the phosphatase towards glycogen (Wang et al., 2002). CBMs are typically found in glucosyl hydrolases and glucotransferases in bacterial, fungal or plant genomes (Boraston et al., 2000; Boraston et al., 2004; Gentry & Pace, 2009; Hashimoto, 2006; Shoseyov et al., 2006). The vast majority of enzymes containing CBMs utilize the domain to bind a specific glucan and enzymatically act on the sugar, as in the case of α -amylase (Boraston et al., 2004). Accordingly, it has been demonstrated that laforin and starch excess 4 protein (SEX4), an *Arabidopsis thaliana* protein with laforin-like properties (Gentry et al., 2007), bind and dephosphorylate glucans, such as glycogen and starch (Gentry et al., 2007; Gentry et al., 2005; Tagliabracci et al., 2007; Tagliabracci et al., 2008; Tagliabracci et al., 2011; Worby et al., 2006). While laforin and SEX4 bind similar types of glucans, they utilize distinct CBMs (Boraston et al., 2004). CBMs are classified into 62 evolutionarily distinct families, based on their primary sequence, secondary and tertiary structure predictions, and crystal structures (Boraston et al., 2004; Hashimoto, 2006; Shoseyov et al., 2006). In 2004, laforin was included in the CBM20 family, formerly known as the starch-binding domain of family 4 - SBD4 (CAZy database - <http://www.cazy.org/Carbohydrate-Binding-Modules.html>) (Coutinho & Henrissat, 1999). At that time, CBM20 family members were only known to be associated with amylases and glucanotransferases in bacteria, fungi and plants (Coutinho & Henrissat, 1999). The degree of homology of laforin and other members of the CBM20 family is very low. Furthermore, the laforin CBM secondary structure does not correlate very well with the other members of the CBM20 family (Girard et al., 2006). Nevertheless, the conservation of three tryptophans is of particular significance. They are invariant both in the few available laforin sequences from other species, as well as in most other CBM20 sequences (Girard et al., 2006).

The phosphatase domain of laforin has been characterized and shown to act as a DSP, based on the differential kinetic parameters of dephosphorylation of the substrates *p*-nitrophenyl phosphate (pNPP) and 3-O-methylfluoresceinyl phosphate (OMFP). For DSPs, the k_{cat}/K_M values are typically more than two orders of magnitude higher with OMFP than with pNPP, whereas they are in the same order of magnitude for protein tyrosine phosphatases (Girard et al., 2006).

The pNPPase activity of laforin has been shown to be inhibited by glycogen and related polysaccharides such as amylopectin and amylose, with inhibition potencies increasing with the decrease in polymer branching (Wang & Roach, 2004). Later, it was observed that laforin can dephosphorylate such polysaccharides (Tagliabracci et al., 2007).

Laforin activity was also shown to be dependent on laforin dimerization via its CBM (Liu et al., 2006) and to interact with proteins involved in glycogen metabolism, such as glycogen synthase (GS) (Worby et al., 2006), and with the glycogen-targeting regulatory subunit R5 of protein phosphatase 1 (PP1) (Fernández-Sánchez et al., 2003). Laforin was also shown to interact with two ubiquitous proteins with unknown functions, EPM2AIP1 (Ianzano, 2003) and HIRIP5, a cytosolic protein predicted to be involved in iron homeostasis (Ganesh et al., 2003). Laforin also interacts with malin, a single subunit E3 ubiquitin ligase containing a RING domain and six NHL domains (Gentry et al., 2005). Malin was shown to interact and to regulate laforin levels by mediating its ubiquitination, and the malin RING domain was necessary and sufficient to mediate laforin polyubiquitination leading to its proteosomal degradation (Gentry et al., 2005). Additionally, laforin was shown to recruit specific substrates

to be ubiquitinated by malin (Lohi et al., 2005; Solaz-Fuster et al., 2008). GS and R5 ubiquitination promote the proteasomal degradation of both proteins (Lohi et al., 2005; Solaz-Fuster et al., 2008), and K63-linked ubiquitination of AMP-activated protein kinase (AMPK, a serine/threonine protein kinase that acts as a sensor of the cellular energy status) which extends the protein lifetime (Moreno et al., 2010). Laforin also promotes the dephosphorylation of glycogen synthase kinase 3 (GSK3) at Ser9, activating this enzyme and leading to the GS phosphorylation at multiple sites with subsequent inhibition (Lohi et al., 2005).

Therefore, laforin seems to be involved in glycogen metabolism as a sensor of poorly branched glycogen resulting from GS overactivity relative to glycogen-branching enzyme activity by modulating GS, either via GSK3 dephosphorylation resulting in GS inhibition, or via malin interaction, resulting in GS ubiquitination and subsequent degradation and inhibition of R5-induced glycogen synthesis.

The laforin/malin complex has also been recently implicated in autophagy, acting as a cellular toxicity suppressor by clearing misfolded proteins through the proteasome system (Aguado et al., 2010; Garyali et al., 2009; Knecht et al., 2010). A putative role for laforin in Tau hyperphosphorylation (one of the distinctive hallmarks of Alzheimer's disease) via GSK3 dephosphorylation has also been reported (Puri et al., 2009).

Despite the solid evidence for laforin's involvement in glycogen metabolism and Lafora disease, little is known about the structural mechanism of the glycogen interaction. This is due to the lack of a solid protocol for producing the large amounts of protein needed for structural studies. In this work we report the expression, purification and characterization of both laforin and its CBM, with appropriate yields for future structural and biophysical studies.

2. Heterologous protein expression and purification

In order to produce enough protein to perform structural studies, heterologous systems must be used. *E. coli* is by far the most widely employed host for heterologous protein expression (Rai & Padh, 2001). Its popularity is due to the vast body of knowledge about its genetics, physiology and complete genomic sequence, which greatly facilitates gene cloning and cultivation (Rai & Padh, 2001). High growth rates combined with the ability to express high levels of heterologous proteins, i.e., strains producing up to 30% of their total protein as the expressed gene product, result in high volumetric productivity. Furthermore, *E. coli* can grow rapidly to high densities in simple and inexpensive media (Rai & Padh, 2001).

Laforin cDNA, coding for full-length laforin with a hexahistidine tag at its C-terminus, was cloned into the vector pET21a by Dr. Jack Dixon's laboratory (Gentry et al., 2005). Previously, this tag was associated with problems in laforin purification and stability (Girard et al., 2006; Liu et al., 2006; Wang & Roach, 2004). Therefore, the hexahistidine tag was removed by using site-directed mutagenesis to introduce a stop codon at the end of the full-length laforin coding sequence. This was achieved using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and using the oligonucleotide primers Laf_ΔHis-for and Laf_ΔHis-rev, whose sequences are presented in Table 1.

The isolated CBM of laforin (Fig. 1, bold sequence) was also produced in order to study the biophysical interaction between the laforin CBM and carbohydrates and for future biotechnology applications. The cDNA sequence of the CBM domain was PCR amplified from the construct provided by Dr. Jack Dixon, using the oligonucleotides Laf_CBM-for and Laf_CBM-rev (Table 1), containing the restriction endonuclease recognition sites for

NcoI and XhoI, respectively, and ligated into NcoI/XhoI linearized pET29a. The Laf_CBM-rev primer introduced a RGD motif followed by a hexahistidine tag at the C-terminus of the CBM. The RGD motif is found in the extracellular matrix and blood proteins, such as fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, and von Willebrand factor and has been described as the major functional group responsible for cellular adhesion (Hwang et al., 2007; Ruoslahti & Pierschbacher, 1987). The inclusion of a RGD motif at the C-terminus of the laforin CBM was intended to be a cell attachment motif in starch-based biomaterial functionalized with the CBM-RGD protein (Moreira et al., 2010).

| Primer name | Primer sequence |
|--------------|--|
| Laf_ΔHis-for | CGTTCTTCTGTGTGTAGCCTGTGAGAGCACCACCACCACCAC |
| Laf_ΔHis-rev | GTGGTGGTGGTGGTGGTGTCTCTCACAGGCTACACACAGAAGAACG |
| Laf_CBM-for | CATGCCATG <u>GGG</u> GATGCGCTTCCGCTTGGGG |
| Laf_CBM-rev | CCGCTCGAGATCACCTCTCATGGTTGGTGGCCTGC |

Table 1. Oligonucleotide primers used for laforin site-directed mutagenesis and for CBM cloning. The stop codon sequence introduced by site-directed mutagenesis is highlighted in bold. Underlined nucleotides indicate the restriction endonuclease recognition sequences used for cloning.

Full-length Laforin and its CBM were expressed by transforming competent *E. coli* BL21 star strain (Invitrogen; a bacterial strain that is suited for high-level recombinant protein expression, due to improved stability of mRNA and the absence of lon and ompT proteases) with the constructs described above. After transformation, cells were plated onto Luria Broth (LB)-agar plates containing the appropriate antibiotic for positive clone selection, and positive colonies were then grown overnight in liquid LB/antibiotic media. These cultures were used to seed typically 1-2 liters of LB/antibiotic and grown at 37 °C until the bacterial culture reached a cell density corresponding to an OD₆₀₀ of 0.6. At that point, protein expression was induced for 3 h by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM.

As previously reported, laforin expression in *E. coli* is associated with the appearance of inclusion bodies (Girard et al., 2006), with the CBM behaving in a similar way (Moreira et al., 2010). Our expertise in refolding proteins expressed in the form of inclusion bodies was crucial for the successful production of high amounts of correctly folded protein (Castanheira et al., 2005; Turner et al., 2001; Simões et al., 2007). After cell pellet disruption, the inclusion bodies were washed in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, followed by a second washing step in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4, 0.1% Triton X-100 (v/v). These washing steps were needed to remove membrane proteins and other contaminants that are released upon cell disruption (Clark, 2001). Purified inclusion bodies were dissolved in 8 M urea buffer (8 M urea, 0.1 M Tris, 1 mM glycine, 1 mM EDTA, pH 10.5) with 100 mM β-mercaptoethanol.

Protein refolding was promoted by rapid dilution (20x) of the dissolved protein into 1 L of 20 mM Tris base, 0.5 mM oxidized/1.25 mM reduced glutathione, 0.5 mM DTT; the pH was then slowly adjusted to pH 8.0 with 6 M HCl solution, and the protein was kept at 4 °C until purification. After 3-7 days in a cold room, the refolded proteins were purified by two


```
1  atgcgcttccgcgttttgggggtggtggtgccacccgccgtggccggcgcccgccgggagctg
   M R F R F G V V V P P A V A G A R P E L
61  ctgggtggtgggggtcgcgggcccgagctggggcggttgggagccgcgcgggtgccgtccgcctg
   L V V G S R P E L G R W E P R G A V R L
121 aggccggccgggcaccgcggcgggcgacggggccctggccctgcaggagccggggcctgtgg
   R P A G T A A G D G A L A L Q E P G L W
181 ctcggggaggtggagctggcgggccgaggaggcggcgcaggacggggcggagccggggccgc
   L G E V E L A A E E A A Q D G A E P G R
241 gtggacacgttctggtacaagtctcctgaagcgggagccgggaggagagctctcctgggaa
   V D T F W Y K F L K R E P G G E L S W E
301 ggcaatggacctcatcatgaccgttgctgtacttacaatgaaaacaacttggtggatggt
   G N G P H H D R C C T Y N E N N L V D G
361 gtgtattgtctcccaataggacactggattgaggccactgggcacaccaatgaaatgaag
   V Y C L P I G H W I E A T G H T N E M K
421 cacacaacagacttctatttttaatatattgcaggccaccaagccatgcattattcaagaatt
   H T T D F Y F N I A G H Q A M H Y S R I
481 ctaccaaatatctggctgggtagctgccctcgtcagggtggaacatgtaaccatcaaactg
   L P N I W L G S C P R Q V E H V T I K L
541 aagcatgaattggggattacagctgtaatgaatttccagactgaatgggatattgtacag
   K H E L G I T A V M N F Q T E W D I V Q
601 aattcctcaggctgtaaccgctacccagagcccatgactccagacactatgattaaacta
   N S S G C N R Y P E P M T P D T M I K L
661 tatagggaagaaggcttggcctacatctggatgccaacaccagatatgagcaccgaaggc
   Y R E E G L A Y I W M P T P D M S T E G
721 cgagtacagatgctgccccaggcggtgtgcctgctgcatgcgctgctggagaagggaacac
   R V Q M L P Q A V C L L H A L L E K G H
781 atcgtgtacgtgcactgcaacgctgggggtgggcccgtccaccgcggctgtctgcggctgg
   I V Y V H C N A G V G R S T A A V C G W
841 ctccagtatgtgatgggctggaatctgaggaagggtgcagtatttcctcatggccaagagg
   L Q Y V M G W N L R K V Q Y F L M A K R
901 cgggctgtctacattgacgaagaggccttgggcccgggcacaagaagatTTTTTccagaaa
   P A V Y I D E E A L A R A Q E D F F Q K
961 tttgggaagggttcgttcttctgtgtgtagcctgtga
   F G K V R S S V C S L -
```

Fig. 1. Laforin cDNA sequence (lower case letters) and translated protein sequence (upper case letters). The amino acids corresponding to the carbohydrate-binding module (CBM) are in bold.

consecutive chromatographic steps. Each refolded protein was first concentrated to 150 mL by tangential flow ultrafiltration (Sartocon Slice, Sartorius), followed by concentration to 12-15 mL with a N₂-pressurized stirred-cell concentrator (Amicon 8200, Millipore). The protein solution was then clarified by ultracentrifugation (100,000x g, 20 min, 4 °C), and the supernatant was applied to a size-exclusion chromatography column (HiLoad 26/60 Superdex 200 prep grade, GE Healthcare), which was previously equilibrated in 20 mM Tris, 0.4 M urea, pH 8.0, at 2 mL/min in order to separate the aggregated forms from the non-aggregated forms of each protein (Fig. 2A and D). Full-length laforin (Fig. 2A) was shown to elute in two separate peaks. The first peak eluted at the expected column void volume (around 115 mL) and corresponded to soluble protein aggregates, and the second peak eluted at around 188 mL, which according to the column calibration should correspond to a globular protein with a molecular weight of 74 kDa, the expected value for the full-length

laforin dimer (2×37 kDa). The fractions obtained during size-exclusion purification were analyzed for phosphatase activity in 50 mM Tris/HCl buffer, pH 7.2 at 30 °C using 10 mM pNPP as the substrate by spectrophotometrically following the increase of absorbance at 410 nm induced by the release of *p*-nitrophenol ($\epsilon_{410\text{nm}} = 18.3 \text{ mM}^{-1} \text{ cm}^{-1}$) (Girard et al., 2006). The second peak from the size-exclusion was active. The active fractions were combined, and the protein was further purified by anion-exchange chromatography on a Mono Q 5/50 GL (GE Healthcare) (Fig. 2B).

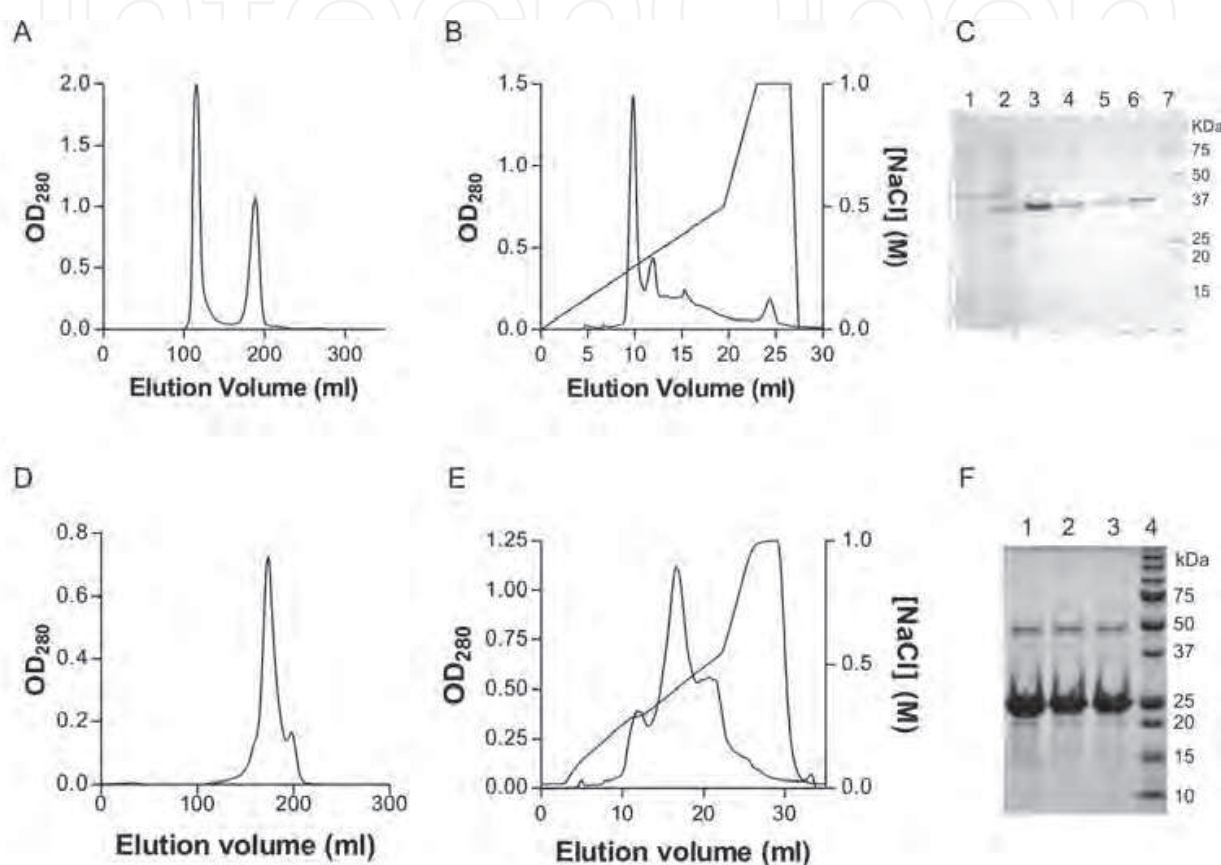


Fig. 2. Protein purification. (A) Full-length laforin size-exclusion chromatogram; (B) Anion-exchange chromatogram of full-length laforin; (C) SDS-PAGE analysis of full-length laforin purification: (1) *E. coli* cells before induction; (2) *E. coli* cells after a 3 h induction, soluble fraction; (3) cells after a 3 h induction, insoluble fraction; (4) concentrated protein applied to a size-exclusion column; (5) second peak from the size exclusion column applied to an anion-exchange column; (6) Anion-exchange major peak; (7) molecular weight standard. The gel was stained with Coomassie brilliant blue. (D) Laforin's CBM size-exclusion chromatogram; (E) Anion-exchange chromatogram of laforin's CBM; (F) SDS-PAGE analysis of laforin's CBM purification: (1) sample applied to a size-exclusion column; (2) second peak (eluted at about 150–200 mL) from the size-exclusion column; (3) highest peak (eluted at about 20 mL) from the anion-exchange column; (4) molecular weight standard. The gel was stained with Coomassie brilliant blue.

| Step | Volume (mL) | Total Activity (U) | Total Protein (mg) | Specific Activity (U/mg) | Yield (%) | Purification Factor |
|----------|-------------|--------------------|--------------------|--------------------------|-----------|---------------------|
| Refolded | 7 | 7250 | 39 | 186 | 100 | |
| Superdex | 38 | 5524 | 22 | 251 | 56 | 1.3 |
| Mono Q | 6.5 | 3400 | 7 | 486 | 47 | 2.6 |

Table 2. Full-length laforin purification table. One unit of activity was defined as the activity corresponding to the consumption of 1 μ M substrate (pNPP) in one minute at 30 $^{\circ}$ C.

Most of the protein was eluted in the major peak with a NaCl concentration of approximately 250 mM, which was also the peak where most of the pNPPase activity was found, indicating that most of the protein was correctly folded and relatively homogeneous. At the end of the purification process from several expression batches, we obtained between 5 and 7 mg of purified laforin per liter of expression media. The purification table (Table 2) shows that the starting material after refolding was already relatively pure, as the purification factor was only 2.6, which is again consistent with the lack of contaminant bands in the SDS-PAGE profile of the refolded protein (Fig. 2C, lane 3).

| Step | Total Protein (mg) | Estimated amount of total CBM (mg) | Yield (%) | Purity (%) |
|---------------------------|--------------------|------------------------------------|-----------|------------|
| Cell extract | 306 | 85.7 | 100 | 28 |
| Refolded protein | 106 | 72.1 | 84 | 68 |
| Applied to size exclusion | 43.4 | 34.7 | 40 | 80 |
| Applied to ion exchange | 33 | 29.7 | 35 | 90 |
| Mono Q (major peak) | 12.8 | 11.5 | 13 | 90 |

Table 3. Laforin CBM purification table. Purity was determined by analysis of SDS-PAGE band intensities using the Quantity One software, version 4.6 (BioRad).

For the CBM of laforin, a similar procedure was adopted. The first purification step, by size-exclusion chromatography (HiLoad 26/60 Superdex 200 prep grade, GE Healthcare), was run with the same conditions as for the full-length protein and revealed no significant aggregates. Most of the protein eluted at 173 mL, which corresponds to tetrameric laforin CBM, and a minor fraction that eluted at 198 mL corresponds to dimerized protein (Fig. 2D). The fractions corresponding to the major peak were combined and applied to the anion exchange column (Mono Q 5/50 GL, GE Healthcare), with most of the protein eluted at 400 mM NaCl (Fig. 2E). The SDS-PAGE analysis of the purified protein revealed an intense band with a molecular weight close to the expected value (22 kDa), and even under denaturing conditions, a band corresponding to the dimeric form of the protein was observed (Fig. 2F). This result is in agreement with the previously described resistance of laforin to dimer separation, prior to SDS-PAGE (Liu et al., 2006).

The purification table of laforin CBM purification shows a yield of more than 12 mg of purified protein per liter of expression media. This protein expression and purification method is the first successful strategy to obtain the laforin CBM and will thus allow its study and characterization as an independent unit, as well as its use in biotechnology applications (Moreira et al., 2010).

3. Heterologous protein characterization

3.1 Protein oligomerization state

Laforin has been shown to exist in a dimeric state; the CBM is responsible for this dimerization because its deletion completely abolishes the dimerization and phosphatase activity of laforin (Liu et al., 2006). The oligomerization state of both laforin and its CBM were analyzed by two distinct methods: analytical size-exclusion chromatography and dynamic light scattering (DLS) (Fig. 3).

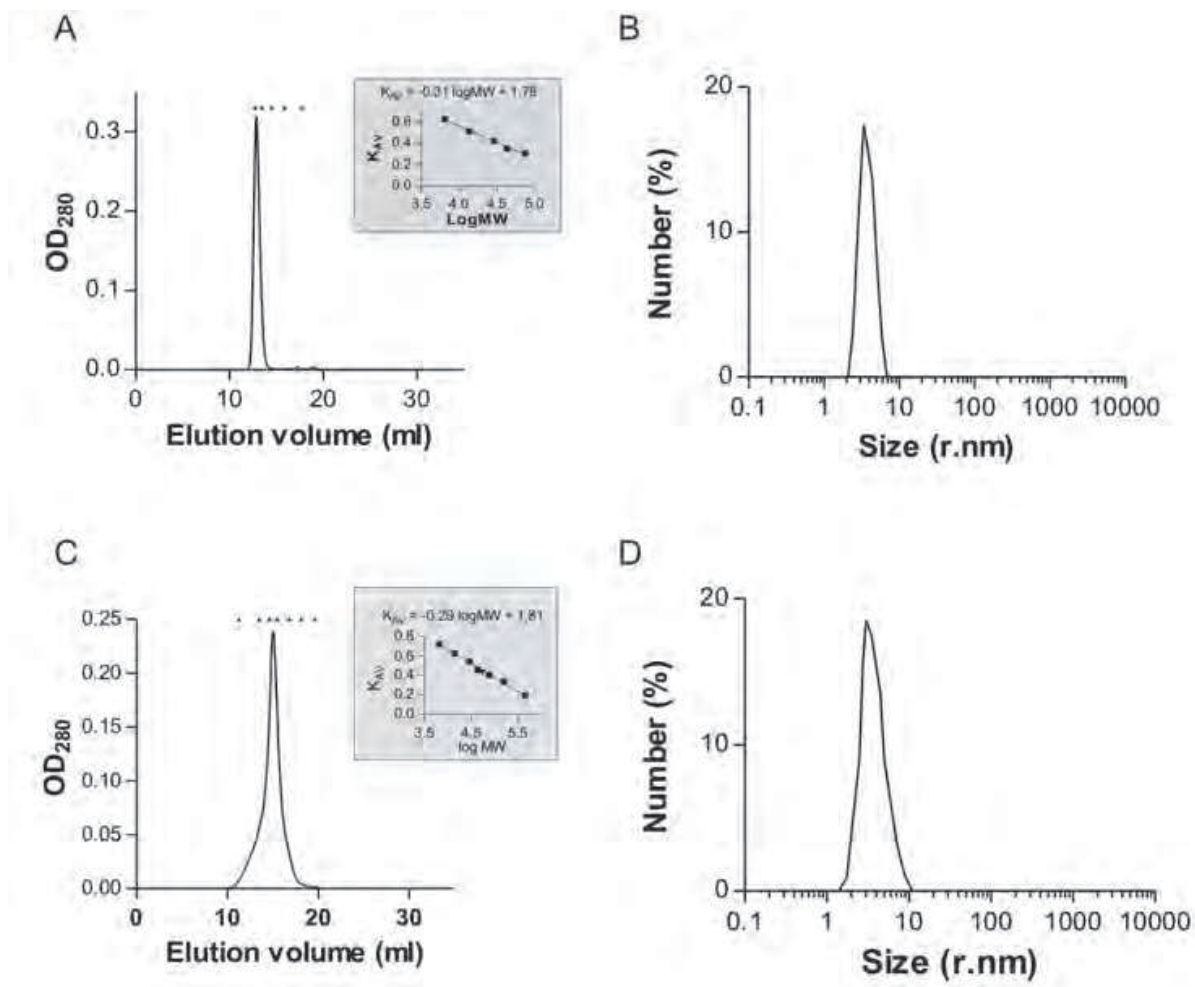


Fig. 3. Protein oligomerization analysis. (A) Analytical size-exclusion chromatography of purified full-length laforin with a Superose 12 10/300 GL column (GE Healthcare); (B) Dynamic Light Scattering analysis of purified full-length laforin; (C) Analytical size-exclusion chromatography of purified laforin CBM with a Superdex 200 10/300 GL column (GE Healthcare); (D) Dynamic Light Scattering analysis of purified laforin CBM.

The analysis of full-length laforin by analytical size-exclusion chromatography on Superose 12 10/300 GL confirmed that laforin is highly homogeneous, eluting with an elution volume corresponding to a globular protein of 64 kDa, based on the column calibration (Fig. 3A, grey box). This result is consistent with the dimer state of full-length laforin (2 x 37 kDa). This result was later confirmed by DLS. The protein was analyzed in both the anion-exchange elution buffer (20 mM Tris, 0.4 M urea, 250 mM NaCl, pH 8.0) and in 50 mM Tris, 50 mM NaCl, pH 7.4, with similar results. The particles detected had a hydrodynamic radius corresponding to a globular protein with a mass of 76 kDa, again consistent with the presence of a dimer.

The analysis of the oligomerization state of the CBM of laforin by analytical size-exclusion chromatography using a Superdex 200 10/300 GL showed that the protein is also in a dimeric state, with a calculated molecular weight of 44 kDa. The differences observed between the preparative size-exclusion chromatography used during protein purification, where the protein eluted as a tetramer, and the analytical size-exclusion chromatography, where the protein eluted as a dimer, are due to the presence of 150 mM NaCl in the analytical size-exclusion chromatography buffer, which was absent from the preparative size exclusion chromatography buffer. The DLS analysis confirmed this result; the protein eluting in the highest peak from the anion-exchange column (Fig. 2E) revealed a hydrodynamic radius corresponding to a 44 kDa protein, thus confirming the dimerization of the isolated protein.

3.2 Protein – carbohydrate interaction assay

The functionality of the CBM, i.e., its ability to bind starch, was evaluated using an adsorption assay, for both the full-length laforin and for the CBM. The purified protein samples were centrifuged (13,000 rpm, 10 min, 4 °C) to remove any precipitated protein, and the protein was then mixed with 50 mg of starch (previously washed with 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 buffer) for 1 h at 4 °C. The mixture was centrifuged (13,000 rpm, 10 min, 4 °C), and the supernatant was analyzed by SDS-PAGE. The starch was washed with buffer (3 x 0.3 mL), and the recombinant protein was eluted from the starch with a 5 mg/mL glycogen solution (0.3 mL, at 4 °C for 1 h). The starch was then treated with buffer containing 2% SDS (0.3 mL, at 95 °C for 5 min) in order to analyze the protein that remained adsorbed after glycogen elution.

The results showed that both full-length laforin and its CBM are able to bind starch and are specifically desorbed from starch when incubated with glycogen. For the full-length laforin, the SDS-PAGE analysis (Fig. 4A) revealed that most of the protein binds to the starch moiety because the protein solution supernatant after starch incubation (Fig. 4A lanes 2) showed a significant decrease in protein levels. This is not due to precipitated protein in the sample because the protein sample was centrifuged prior to the starch incubation. The subsequent washing steps revealed no washed protein (Fig. 4A lanes 3), and after the incubation with glycogen, laforin was effectively eluted from starch (Fig. 4A, lane 4). From the differences in band intensities between the initial protein and the supernatant after incubation, one would expect a stronger band from the glycogen elution, but the relatively faint band resulting from the glycogen elution can be attributed to a stronger binding of laforin to starch rather than glycogen, as previously reported (Chan et al., 2004). The results obtained with the CBM are in line with this assumption because a stronger protein band is observed in the starch pellet after glycogen elution (Fig. 4B, lane 4) than the one resulting from the glycogen elution (Fig. 4B, lane 3).

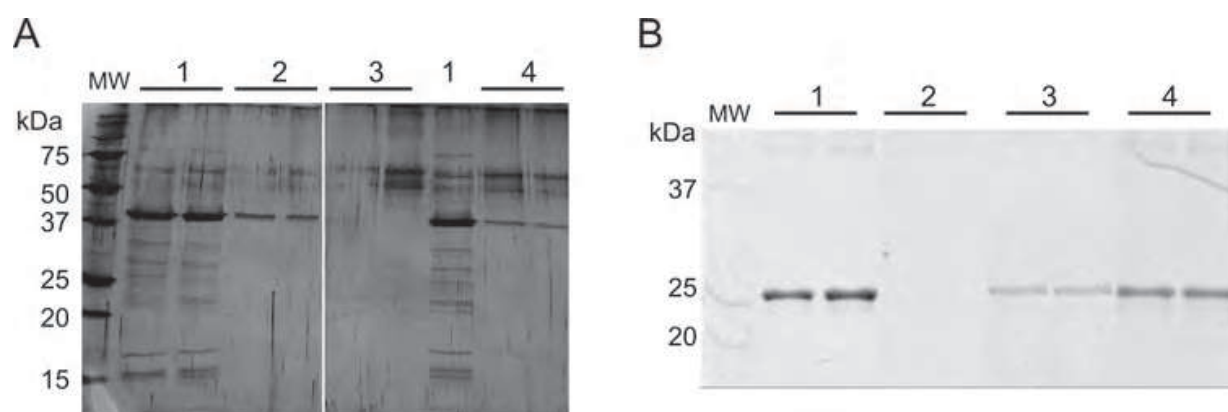


Fig. 4. Protein–starch-binding assay. (A) Full-length laforin starch-binding assay: (1) laforin supernatant before starch incubation; (2) supernatant after starch incubation; (3) starch-washing supernatant; (4) supernatant after glycogen elution. All steps were performed in duplicate. Gel was silver stained. (B) CBM starch-binding assay: (1) CBM supernatant before starch incubation; (2) starch-washing supernatant; (3) supernatant after glycogen elution; (4) starch pellet after glycogen elution. All steps were performed in duplicate. The gel was Coomassie stained.

Together, these results confirmed the starch binding activity of both laforin and its CBM.

3.3 Laforin phosphatase activity characterization

The laforin phosphatase activity was characterized using both pNPP (Montalibet et al., 2005) and OMFP, previously reported to be a better substrate for dual-specificity phosphatases than pNPP (Girard et al., 2006; Gottlin et al., 1996). The kinetic parameters for pNPP as a substrate were measured in a total volume of 1 mL by incubation of the enzyme with various amounts of substrate in 50 mM Tris-HCl buffer, pH 7.2 in a Varian Cary 100 UV/Vis spectrophotometer with the temperature control set to 30 °C by following the absorbance increase at 410 nm induced by the release of *p*-nitrophenol ($\epsilon_{410\text{nm}} = 18.3 \text{ mM}^{-1} \text{ cm}^{-1}$) (Girard et al., 2006). For OMFP, the kinetic parameters were obtained by a fluorescence-based assay, using conditions similar to the ones used for pNPP. The assay was performed using a Horiba Jobin Yvon Fluoromax-3 fluorometer by following the fluorescence emission of the product formed with excitation at 485 nm and emission measured at 530 nm. The data were fit to the Michaelis-Menten equation (Eq. 1) using the software Enzyme Kinetics Module v.1.2 – Sigmaplot v 9.01 (Systat Software, Inc.).

$$V = k_{\text{cat}} [S] / (K_M + [S]) \quad (1)$$

The kinetic parameters (Table 4) show that the recombinant laforin produced in this study has similar properties when compared to the previously published kinetic parameters for GST- and His-tagged versions of laforin (Girard et al., 2006), confirming the behavior of laforin as a dual-specificity phosphatase with OMFP better as the substrate than pNPP (Castanheira et al., 2010).

The pH profile of laforin phosphatase activity was also evaluated using OMFP as a substrate. The results presented in Fig. 5A are in agreement with the results published earlier for tagged versions of laforin (Girard et al., 2006; Peters et al., 2003; Wang et al., 2002; Wang & Roach, 2004), with maximal activity at the acidic pH of 6.0. The capability of

glycogen to inhibit the phosphatase activity of laforin was also evaluated by including increasing amounts of glycogen in the phosphatase activity assay. The results presented in Fig. 5B show that laforin, in agreement with previously published reports (Girard et al., 2006; Wang & Roach, 2004), is effectively inhibited by glycogen with an AC₅₀ of around 50 µg/mL.

| Enzyme | pNPP | | | OMFP | | |
|----------------|-------------------------------------|---------------------|---|-------------------------------------|---------------------|---|
| | k _{cat} (s ⁻¹) | K _M (mM) | k _{cat} /K _M (s ⁻¹ .mM ⁻¹) | k _{cat} (s ⁻¹) | K _M (mM) | k _{cat} /K _M (s ⁻¹ .mM ⁻¹) |
| Laforin | 0.56 ± 0.07 | 8.4 ± 0.7 | 0.07 ± 0.01 | 7.6 ± 1.5 | 0.21 ± 0.05 | 36 ± 11 |
| GST-pG laforin | 1.1 ± 0.1 | 11.4 ± 3.0 | 0.10 ± 0.03 | 9.5 ± 2.0 | 0.26 ± 0.04 | 36 ± 13 |
| His-laforin | 1.7 ± 0.2 | 33 ± 3 | 0.051 ± 0.004 | 6.5 ± 1.3 | 0.75 ± 0.29 | 8.7 ± 5.1 |

Table 4. Steady-state kinetic parameters for laforin phosphatase activity. Comparison with published data for tagged versions of laforin, obtained from Girard (2006).

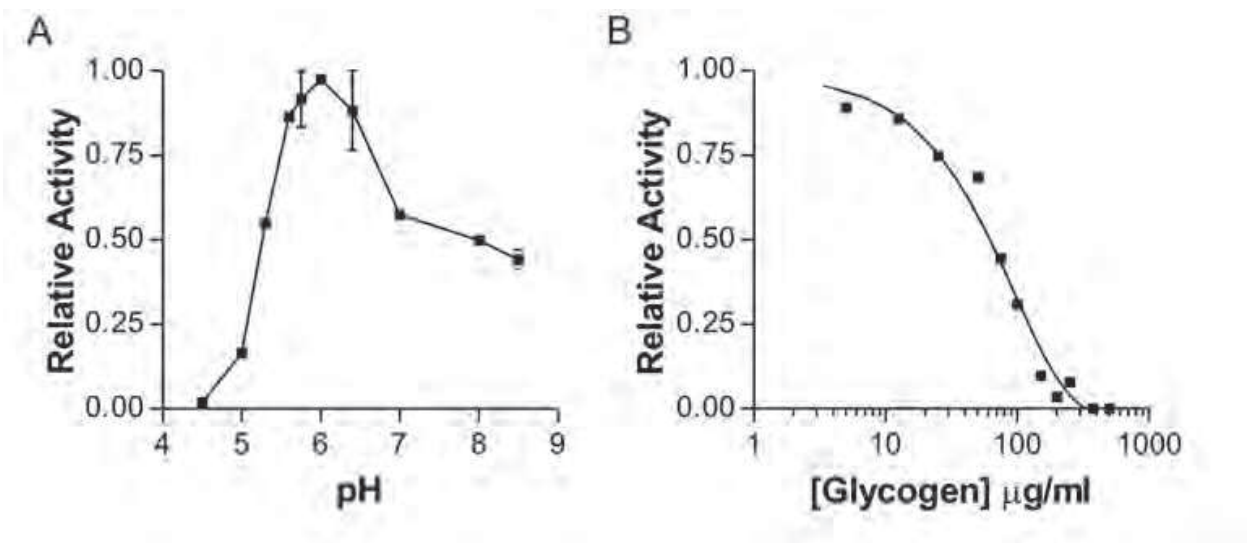


Fig. 5. Laforin phosphatase activity characterization: Optimum pH (A) and inhibition by glycogen (B).

4. Conclusion

In this work, we have shown that both full-length laforin and its CBM can be produced in *E. coli* expression systems in high amounts and purified without the use of fusion tags. The characterization of the purified protein showed that in both cases, the protein is dimerized and has a functional CBM. For full-length laforin, the phosphatase activity was characterized and shown to have similar characteristics to the various forms of laforin previously described. The protein expression methodology used in this study proved to be suited for the production of high amounts of protein. This will enable the structural characterization and determination of the three-dimensional structure of laforin, which will contribute to the understanding of the molecular mechanism of action of laforin. The

understanding of both the mechanism of the laforin-laforin or laforin-malin interactions is important for the development of Lafora disease therapies. The protein-protein and protein-carbohydrate interfaces represent targets for the design of new drugs that promote or stabilize laforin's dimerization or its interaction with binding partners (Veselovsky et al., 2002).

5. Future perspectives

The work presented in this chapter paves the way for the structural studies needed to understand the molecular mechanism of protein-carbohydrate interaction. These studies will be performed using techniques such as NMR that will provide site-specific information on the architecture, binding selectivity and plasticity of the carbohydrate-binding sites of laforin. Other biophysical techniques such as Trp fluorescence have been used to address the chemical polarity shifts associated with protein-carbohydrate interactions. Protein-carbohydrate interactions will also be studied by surface plasmon resonance in order to obtain the kinetic parameters of carbohydrate-ligand binding with wild-type and mutant proteins. Finally, X-ray crystallography and solution NMR will be used to obtain the three dimensional structure of laforin and its CBM, respectively.

These studies will most likely provide new insights into the molecular mechanism of protein-carbohydrate binding and into the structural determinants involved in laforin dimerization, which will create new opportunities for the design of Lafora disease therapies and novel biotechnology applications for carbohydrate-binding proteins.

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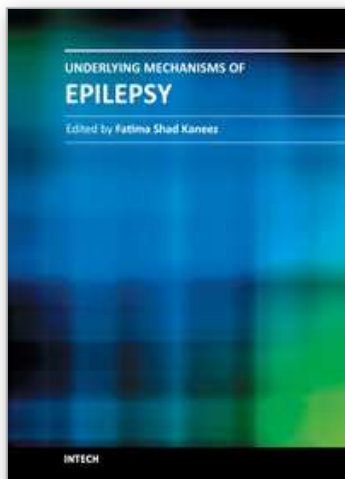
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This book is a very provocative and interesting addition to the literature on Epilepsy. It offers a lot of appealing and stimulating work to offer food of thought to the readers from different disciplines. Around 5% of the total world population have seizures but only 0.9% is diagnosed with epilepsy, so it is very important to understand the differences between seizures and epilepsy, and also to identify the factors responsible for its etiology so as to have more effective therapeutic regime. In this book we have twenty chapters ranging from causes and underlying mechanisms to the treatment and side effects of epilepsy. This book contains a variety of chapters which will stimulate the readers to think about the complex interplay of epigenetics and epilepsy.

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