

Expression of the functional carbohydrate-binding module (CBM) of human laforin

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ABSTRACT

Laforin is a human protein associated with the glycogen metabolism, composed of two structurally and functionally independent domains: a phosphatase catalytic domain and a substrate-binding module with glycogen and starch affinity. The main goal of this work is the development of a methodology for the expression of the so far poorly characterized carbohydrate-binding module (CBM) of laforin, allowing its study and development of biomedical applications. The laforin's CBM sequence was originally cloned by PCR from a human muscle cDNA library. The recombinant protein, containing laforin's CBM fused to an Arg-Gly-Asp sequence (RGD), was cloned and expressed using vector pET29a and recovered as inclusion bodies (IBs). Refolding of the IBs allowed the purification of soluble, dimeric and functional protein, according to adsorption assays using starch and glycogen. Several other experimental approaches, using both bacteria and yeast, were unsuccessfully tested, pointing towards the difficulties in producing the heterologous protein. Indeed, this is the first work reporting the production of the functional CBM from human laforin.

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Introduction

Laforin is a human dual specific phosphatase (DSP),² coded by the EPM2A gene, involved in the glycogen metabolism and implicated with a human disorder – Lafora disease (LD), a progressive myoclonus epilepsy [1–3].

Laforin presents the modular structure frequently found in the CAZymes (enzymes that act on carbohydrates) [4], containing a carbohydrate-binding module (CBM) at the N-terminus connected by a linker to the C-terminal catalytic module [2]. Since each module performs and folds independently, several CBM applications have been described, namely its use as purification and solubilization tags in fusion proteins [5]. CBMs are classified according to the sequence homology, the laforin's CBM being assigned to family 20 (<http://www.cazy.org/>). The three-dimensional (3D) structure of laforin (including its CBM domain) is still unknown. Furthermore, recombinant laforin has been reported to aggregate easily, which

explains the difficulty in obtaining enough protein for 3D structure analyses [6].

It has been shown that this CBM binds complex carbohydrates *in vivo* and *in vitro* [2], and the DSP motif can hydrolyze phosphotyrosine and phosphoserine/threonine substrates *in vitro* [1,6]. Laforin is the single human phosphatase containing a CBM and has been shown to liberate phosphate from the complex carbohydrate amylopectin, whereas other phosphatases lack this activity [7]. The main function of CBMs is to target the enzyme to the substrate, increasing its activity, particularly in the case of insoluble carbohydrates [5]. Besides binding laforin to glycogen, it was also demonstrated that this CBM also drives the enzyme to Lafora Bodies (LBs), dense aggregates of polyglucosan fibrils structurally similar to starch [8]. In addition, the CBM of laforin fused to the DSP of vaccinia H1-related phosphatase still binds amylopectin [7], thus the laforin-CBM remains functional when fused with other peptides. In this context, bioactive peptides may be adsorbed to biomaterials made of starch, through the fusion with CBM, in order to achieve their functionalization for biomedical applications.

Among the bioactive molecules used to functionalize biomaterials, proteins of the extra-cellular matrix (ECM), poly-L-lysine (PLL) and a natural adhesive protein extracted from mussel (MAP) [9] have been successfully applied in promoting cell adhesion and proliferation [10–13]. The Arg-Gly-Asp (RGD) motif – found in ECM and blood proteins, such as fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, and von Willebrand factor – was described as the major functional group responsible for

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² Abbreviations used: CBM, carbohydrate-binding module; RGD, Arg-Gly-Asp sequence; IBs, inclusion bodies; DSP, dual specific phosphatase; LD, Lafora disease; 3D, three-dimensional; LBs, Lafora Bodies; ECM, extra-cellular matrix; PLL, poly-L-lysine; RGD, Arg-Gly-Asp; IMAC, immobilized metal ion affinity chromatography; DLS, dynamic light scattering; CAZy, carbohydrate-active enzymes database; IPTG, isopropyl β-D-1-thiogalactopyranoside.

cellular adhesion [9,14]. Several strategies to functionalize materials, through the immobilization of RGD on the material surface, have been developed [15–17]. However, most of these strategies involve the activation of either the polymer or the RGD-containing sequence, to allow for the covalent binding. On the other hand, it has been described that the RGD bioactivity can be conserved in fusion proteins [18,19]; therefore, in this work, the RGD was fused to the laforin's CBM in order to produce a recombinant protein for functionalization of starch material, by the adsorption of the protein through CBM affinity. Indeed, several CBMs were already used for protein targeting [20,21], including a CBM from family 20 with starch affinity, which was fused with RGD sequence to functionalize starch-based materials [22]. However, the utilization of a human protein seems a better choice when *in vivo* applications are envisioned.

Although laforin has been cloned and purified, the application of the laforin-CBM as a target partner has never been described. Therefore, in this study, a strategy was developed to express, purify and functionally characterize a recombinant protein containing the human laforin-CBM fused to RGD tripeptide in *Escherichia coli*.

Material and methods

Reagents and strains

All reagents used were laboratory grade from Sigma–Aldrich (St. Louis, USA), unless stated otherwise. *E. coli* strain XL1 Blue, from Stratagene (Carlsbad, CA, USA) was used as bacterial host for DNA cloning. For protein expression *E. coli* BL21 (DE3) strain and the T7 plasmids pET29a and pET25b (+) were purchased from Novagen (Madison, USA) and pGEX4-T1 from GE Healthcare. The oligonucleotides presented in Table 1 were purchased from MWG Biotech (Germany). Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostics GmbH (Penzberg, Germany). Pfu DNA polymerase used was from Stratagene, and the MasterAmp 10X PCR Enhancer from EPICENTRE Biotechnologies. Thrombin protease and isopropyl β -D-1-thiogalactopyranoside (IPTG) were from GE Healthcare. The theoretical molecular masses of the recombinant proteins were calculated using the Compute pI/Mw application from ExpASY Proteomics Server (<http://www.expasy.ch/tools>).

Gene cloning

The DNA coding sequence of the glycogen-binding module of laforin was amplified using a human muscle cDNA library (CLONTECH). This sequence was used as template, to clone the CBM coding sequences by PCR with the RGD coding sequence being introduced in the reverse primer. The PCR reactions were performed using Pfu DNA polymerase (2.5 U), 0.5 mM of each primer (forward and reverse according to Table 1), 1.2 mM MgSO₄, 0.24 mM dNTP, 1.2X enzyme buffer, and 1.2X of PCR enhancer solution. PCR conditions were: denaturation at 95 °C, annealing at 56 °C and extension at 72 °C, all steps for 45 s (this cycle was repeated 30 times).

The DNA coding sequences were cloned in different expression systems, allowing for the fusion of recombinant proteins with a hexa-histidine tag (6xHis) on the C-terminal (pET expression systems) or GST on the N terminal (pGEX expression system), for purification. The *E. coli* XL1 Blue was used as cloning strain and expression was carried out in *E. coli* BL21 (DE3), *E. coli* BL21 star, Origami or Tuner. Cell carrying the non-modified plasmid (without DNA coding sequence) was used as negative control.

The integrity of cloned PCR products was verified by DNA sequencing [23] using ABI PRISM310 Genetic Analyzer.

Recombinant His-tagged protein expression and purification

The non-modified pET25, pET25-CBM or pET25-CBM-RGD was used to transform *E. coli* expression host (BL21 DE3, Origami, Tuner). Cells were cultivated in LB medium at 30 °C until OD₆₀₀ reached 0.4 and induced by adding IPTG to a final concentration of 0.1 mM for 16 h. At the end of fermentation, cells were harvested and periplasmic proteins recovered using the previously described protocol [24].

To recover the cytoplasmatic proteins, cells were lysed in buffer containing 50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 0.1% β -mercaptoethanol, 1 mM PMSF. After sonication, 0.6 M arginine and 1% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) were added to the lysate. The mixture was incubated at 4 °C, with gentle agitation for 16 h and centrifuged (30 min, 15 000 rpm, 4 °C). The recombinant protein in the supernatant was purified by immobilized metal ion affinity chromatography (IMAC), using 5 ml Nickel His-Trap columns (GE Healthcare) according to the manufacturer's instructions.

Expression and purification of GST-tagged recombinant protein

Cells transformed with expression vectors pGEX-CBM, pGEX-CBM-RGD or non-modified pGEX were grown at 37 °C in LB medium until OD₆₀₀ reached 0.7. At this point temperature was lowered to 20 °C and after 1 h protein expression was induced by IPTG addition (0.1 mM final concentration). twenty hours later, cells were harvested, resuspended in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) and incubated with deoxyribonuclease I (100 μ g/ml) and MgCl₂ (100 mM). After DNA digestion, 100 μ l of Triton X-100 was added and the supernatant incubated with Glutathione–Sepharose CL 4B (GE Healthcare). Protein elution was performed using Tris buffer (50 mM Tris–HCl, pH 8.0) containing 10 mM reduced glutathione. Recombinant protein was treated with thrombin protease (10 U/mg of recombinant protein), according to the manufacturer's instructions (GE Healthcare).

Recombinant protein expression in inclusion bodies (IBs), refolding, and purification

The culture of *E. coli* BL21 star (Invitrogen) transformed with pET29a-CBM-RGD expression vector was grown in 1 L LB medium with 30 μ g/ml kanamycin at 37 °C, 185 rpm in New Brunswick Innova 44R incubator shakers. The expression of CBM-RGD protein

Table 1

Primers utilized to amplify the coding sequence in the different expression systems. The sequences recognized by the restriction enzymes are in bold. The RGD coding sequence is underlined.

| Plasmid | Primer (5' → 3') | Restriction enzyme |
|-------------------|--|--------------------|
| pET25b and pET29a | For CATG CCATGGGG ATGCGCTTCCGCTTTGGGG | NcoI |
| | Rev GGAATTC ATGGCTTGGTGGCCTGC | EcoRI |
| | Rev CCG CTCGAGATCACCTC TCATGGTTGGTGGCCTGC | XhoI |
| pGEX 4T1 | For GGATCCATGCGCTTCCGCTTTGGGG | BamHI |
| | Rev GGAATTC ATGGCTTGGTGGCCTGC | EcoRI |
| | Rev CCG CTCGAGATCACCTC TCATGGTTGGTGGCCTGC | XhoI |

was induced by the addition of IPTG into the culture medium at mid-log phase (OD_{600} 0.6) to 0.5 mM final concentration. After 3 h, 3.74 g of cells (wet weight) were harvested, resuspended in 50 ml of buffer A (50 mM Tris–HCl, 50 mM NaCl, pH 7.4), and lysed by adding lysozyme (100 μ g/ml). After freezing and thawing, deoxyribonuclease I (100 μ g/ml) and $MgCl_2$ (100 mM) were added and incubated at 4 °C for 1 h. The IBs were then washed for 3 h with 1 L of buffer A, centrifuged (at 10 000g for 20 min at 4 °C), and washed again for 3 h with 1 L of buffer A containing 0.1% Triton X-100 (v/v). Upon centrifugation (10 000 g for 20 min at 4 °C), purified IBs were dissolved in 50 ml of 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 and then the protein was refolded by rapid dilution (20-fold) at room temperature into 1 L of 20 mM Tris, 0.5 mM oxidized/1.25 mM reduced glutathione, 0.5 mM DTT without prior pH adjustment; the pH was then slowly (over a 2 h period) adjusted to 8.0 with 6 M HCl and the solution was kept at cold room until purification.

After 3–4 days to a week period the refolded CBM-RGD solution was first concentrated by tangential flow ultrafiltration (Pellicon 2; Millipore) to approximately 150 ml, followed by a N_2 pressurized stirred cell concentrator (Amicon 8200 – Millipore) to 12–15 ml. After ultracentrifugation to clarify the solution (100 000 g, 20 min, 4 °C), the protein was applied to a 320–330 ml bed volume HiLoad 26/60 Superdex 200 prep grade column (Amersham) pre-equilibrated at room temperature with 20 mM Tris, 0.4 M urea, pH 8.0 at 2.0 ml/min. Urea was kept in the purification buffer in order to prevent the protein aggregation [25].

The fractions eluted between 150 and 200 ml, corresponding to non-aggregated forms of recombinant protein, were then combined and further purified by ion exchange chromatography on a 1 ml Mono Q HR 5/5 column (GE Healthcare) using the same buffer as for the Superdex 200 chromatographic experiment with a linear gradient of NaCl (0–0.5–1 M) at 0.75 ml/min.

Recombinant protein analysis

Recombinant protein was analyzed either by Coomassie stained 12% SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and 10% native PAGE, which was used to evaluate protein aggregation. For MW determination the protein was analyzed by gel filtration chromatography on a 24 ml bed volume Superdex 200 10/300 GL column (GE Healthcare) equilibrated in 20 mM Tris–HCl, 150 mM NaCl, pH 8.0 buffer, at 0.4 ml/min. The molecular size and weight of the protein were also estimated by dynamic light scattering (DLS; Nonozetasizer, Malvern). The DLS measurements were performed at room temperature, using a protein sample at 0.5 mg/ml in the same buffer (20 mM Tris, 150 mM NaCl, pH 8.0).

Adsorption assay

To evaluate the human laforin-CBM substrate affinity and specificity, adsorption assays using starch were carried out. The purified protein samples (0.25 mg/ml) were centrifuged (13 000 rpm, 10 min, 4 °C) to remove any precipitated protein, and then the protein was 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 analyzed by SDS–PAGE. The starch was washed with buffer (3X 0.3 ml) and the recombinant CBM was eluted from 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.

Results

Recombinant laforin's CBM expression and purification

Several strategies were used, attempting the production of the soluble CBM, including periplasmic secretion (pET25b) and fusion with GST – a solubility enhancer tag. Fig. 1 summarizes these approaches and the main results obtained under the conditions tested. Using pET25b, the recombinant protein was not detected in the periplasmic fraction under the conditions tested. The protein was rather expressed in the cytoplasmic fraction and only in the presence of arginine and CHAPS the purification was possible, using the IMAC system. Nevertheless, native PAGE and DLS analyses indicated protein aggregation and the adsorption assay showed that CBM was not functional (data not shown).

When fused to GST, although soluble, the purified protein was obtained in very low quantities and the CBM did not present starch affinity (data not shown).

Finally, the solubilization and refolding of IBs was the approach leading to the functional CBM; only those results will be presented in this work.

Escherichia coli BL21 star cells transformed with pET29a-CBM vector were grown in 1 L of LB medium at 37 °C and induced with 0.5 mM IPTG for 3 h. Table 2 summarizes the results obtained using the IBs refolding protocol.

Fig. 2 shows the SDS–PAGE analysis of the protein obtained in the insoluble fraction and, after the washing step, recombinant protein showed 68% purity (Fig. 2C, lane 1). The protein has an apparent molecular weight close to the expected (22 kDa) and even under denaturing condition is possible to detect the dimeric form (44 kDa). This result is in agreement with the previously described resistance of laforin to dimer separation, prior to SDS–PAGE [26]. During the refolding step, about 40% (62.6 mg) of protein had precipitated, but the insoluble material was successfully removed by ultracentrifugation prior to its application onto HiLoad 26/60 Superdex 200 column. The protein detected on the major peak (Fig. 2A), corresponding to the tetramer form of the protein, was loaded onto Mono Q column in order to separate the different protein conformation (Fig. 2B). The purified protein was then analyzed and the results from Superdex 200 10/300 GL column suggested that protein is a dimer. The difference in the oligomerization state between the preparative and analytical Superdex 200 columns resides on the fact that the protein is converted into a dimer in the presence of 150 mM of NaCl, which is not present during preparative purification (data not shown).

Native PAGE analysis and gel filtration chromatography (Superdex 200 10/300 GL equilibrated with 20 mM Tris–HCl, 150 mM NaCl, pH 8.0 buffer) of the highest peak, collected from Mono Q column (Fig. 2B and C, lane 3) showed that the refolded protein was not aggregated and exhibits the size corresponding to the dimeric form (44 kDa) and is highly purified (Fig. 3A and B). In addition, the DLS analyses of the sample (in buffer containing 150 mM of NaCl) detected a particle with hydrodynamic diameter of 6.5 nm, corresponding to a 44 kDa protein (Fig. 3C) using an empirical calibration developed by Malvern Instruments, thus confirming the dimerization of the isolated protein. The polydispersity of the sample was 0.15% and the result shown corresponds to an average of 10 measurements.

The ability of the recombinant laforin's CBM to bind carbohydrates

The functionality of the recombinant protein, i.e., its ability to bind starch, was evaluated using an adsorption assay. Briefly, the protein was incubated with starch and after 1 h, three washing steps were performed with buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.4

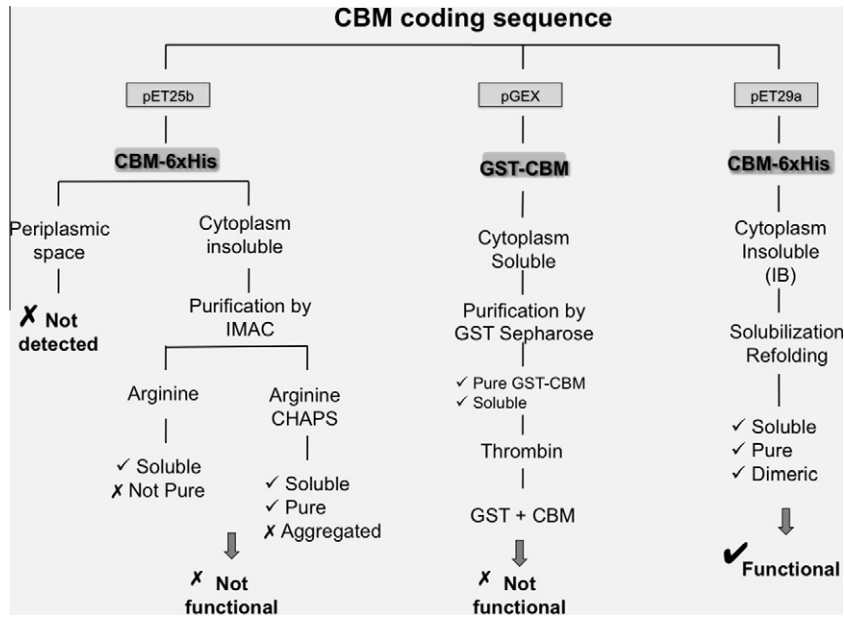


Fig. 1. Summary of strategies and results obtained under the conditions tested.

Table 2
Purification table.

| | Total protein (mg) | Estimated amount of total CBM | Yield (%) | Purity (%) |
|------------------|--------------------|-------------------------------|-----------|------------|
| Cell extract | 306 | 85.7 | 100 | 28 |
| Refolded protein | 106 | 72.1 | 84 | 68 |
| Applied to Sx200 | 43.4 | 34.7 | 40 | 80 |
| Sx200 #15–18 | 33 | 29.7 | 35 | 90 |
| Mono Q #9–14 | 12.8 | 11.5 | 13 | 90 |

The starting material was 1 L of *E. coli* expressing CBM-RGD (weight of wet bacteria: 3.74 g). Purity was determined by analysis of SDS–PAGE band intensities using the Quantity One software, version 4.6 (BioRad).

buffer). Since no protein has been removed (Fig. 4 – lanes W1 and W2), the elution was performed by incubating the starch moiety with a glycogen (5 mg/ml) containing buffer. After 1 h, the sample was centrifuged and both the supernatants (E1 and E2) and the pellets (P1 and P2) were analyzed by SDS–PAGE. The results showed that the glycogen was effective in promoting the desorption of CBM, although not completely, since most of the CBM was still present in the pellet fraction (Fig. 4 – lanes P1 and P2).

Discussion

This is the first report on the expression of the unusual laforin-CBM. The production of functional CBM, now achieved, will allow the structural characterization, by crystallography, contributing for the elucidation of mechanistic aspects related to the Lafora disease. Several biomedical applications can be envisioned using this human CBM, such as tag molecule for adsorbing small bioactive peptides to starch-based biomedical materials. The expression of soluble and functional heterologous proteins may be a difficult task, especially for eukaryotic proteins [27]. The heterologous bacterial expression systems remain the most attractive ones due to low cost, high productivity, the well-known genetics and the large number of compatible molecular tools available [28,29]. Usually, overexpressed recombinant proteins accumulate either in the cytoplasm and/or in the periplasmic space. However, overexpression of recombinant proteins in bacterial hosts frequently results in IBs, which are amorphous granules of misfolded protein with no biological activity [28,30–32]. The IBs formation frequently occurs, when overexpressing eukaryotic proteins, since post-translation modification processes are often required for their correct folding and functionality [27,33,34]. Commonly, the protein

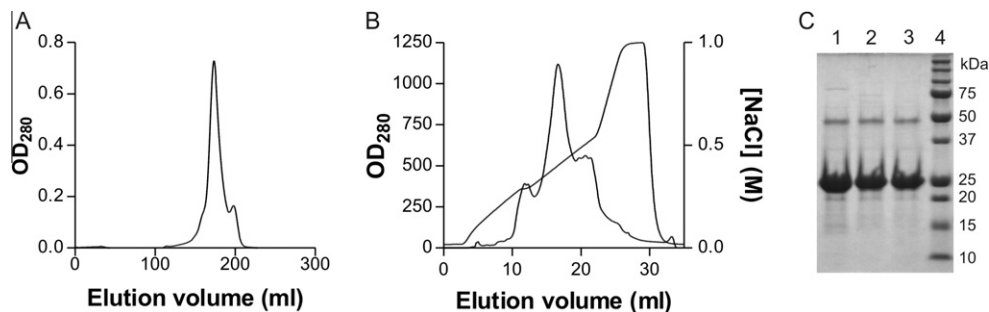


Fig. 2. CBM-RGD chromatographic purification. (A) The refolding, after concentrated, solution was applied to a HiLoad 26/60 Superdex 200 column pre-equilibrated with 20 mM Tris, 0.4 M urea, pH 8.0; (B) the fraction from the second protein peak from Superdex 200 were loaded onto a 1 ml Mono Q column equilibrated with 20 mM Tris, 0.4 M urea, pH 8.0. Elution was done by a gradient of NaCl; (C) SDS–PAGE analysis: (1) Sample applied to Superdex 200; (2) Second peak (eluted at about 150 – 200 ml) from Superdex 200; (3) Highest peak (eluted at about 20 ml) from Mono Q; (4) Molecular weight Standard.

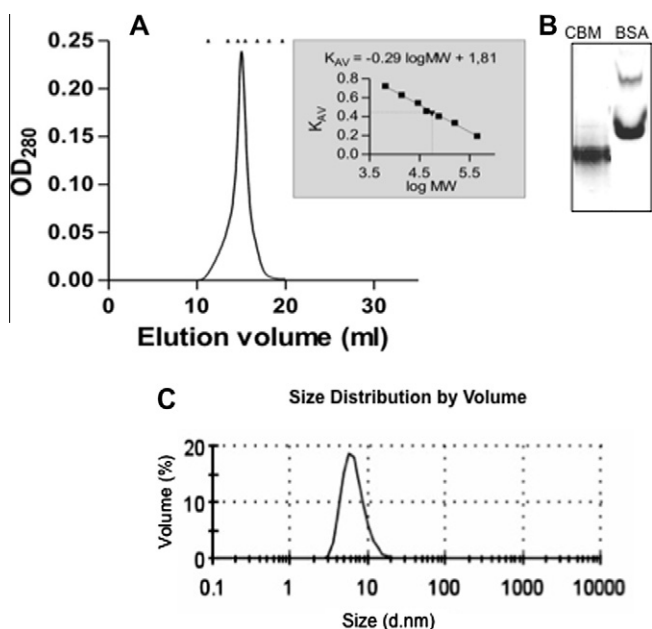


Fig. 3. Analysis of the protein by (A) gel filtration chromatography using a Superdex 200 10/300 GL equilibrated with 20 mM Tris, 150 mM NaCl, pH 8.0 buffer that had been previously calibrated using the GE Healthcare gel filtration molecular weight calibration kit (highlighted box), the triangles indicate the elution volumes of MW markers used for column calibration, (B) native PAGE and (C) DLS analysis of purified protein in buffer containing 150 mM of NaCl. BSA was used as control.

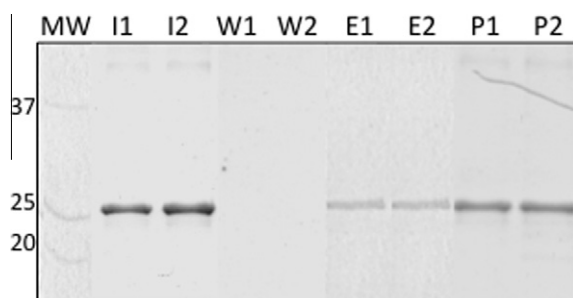


Fig. 4. SDS-PAGE of CBM-RGD adsorption assay to starch. Initial protein (I), washing fraction (W), elution fraction of CBM-RGD with buffer containing glycogen (E) and pellet (P). All steps were done in duplicate and using different protein stock preparations.

expression in IBs is not a first choice for protein production due to poor recovery yields and the customization of the refolding conditions for each target protein. Furthermore, the re-solubilization procedures may affect the integrity of the refolded proteins [27,28]. However, in this study, the functional CBM could be obtained only through IBs solubilization and refolding.

Several strategies to enhance the solubility of recombinant proteins are available, among them the use of fusion tags and optimization of the growth and induction conditions [35–37]. In this work, several expression systems were used, attempting to produce soluble CBM, including the fusion with GST-tag, secretion to periplasmic space (where protein was expected to be mostly soluble and correctly processed), different expression hosts, different growth and induction conditions; nevertheless, the recombinant CBM was always obtained in the insoluble fraction or, when soluble, aggregate, lacking starch affinity (data not shown). Moreover, results using chaperone co-expression and *Pichia pastoris* expression systems (unpublished results) also lead to aggregated recombinant CBM. The full protein laforin, comprising the two modules, has been expressed by other authors as a fusion protein

(with GST, 6x-His) in *E. coli*. This protein showed phosphatase activity on model substrate and affinity for glycogen and starch [1–3,6,38,39]; however, laforin was described as aggregating easily, and indeed it presents different solubility depending on the fused tag [6], with 6xHIS-tagged laforin being less soluble than GST-laforin.

Taken together, the results obtained with the different expression systems point towards the CBM domain being less soluble than the full-length laforin. Indeed, the same expression system effective for the production of the soluble full-length laforin is not suitable for CBM alone.

It is known that, *in vivo*, the dimerization of laforin, through a CBM–CBM interaction, is essential for its phosphatase activity [26,40]. The protein obtained by inclusion bodies solubilization and refolding presented different oligomerization state depending on the presence of salt in the buffer. The DLS analyses of recombinant protein, in buffer containing 150 mM of NaCl, indicated that protein presented a hydrodynamic diameter of 6.5 nm, corresponding to a protein with 44 kDa. The DLS analyses confirm the gel filtration chromatography results, suggesting that the protein obtained is the pure CBM dimer, in buffer containing 150 mM of NaCl. Furthermore, the CBM adsorbed to starch was partially eluted using glycogen, indicating its functionality; however, a considerable amount of CBM remained in the starch-pellet, probably due to the stronger binding of laforin towards starch than glycogen, as previously reported [8] or to the presence of inactive material that precipitated on starch particles.

Further work including assays using CBM-RGD on dextrin hydrogel, as described in a previous work for bacterial CBM [22], and the evaluation in cell adhesion and proliferation on biomaterial; the production of novel recombinant proteins containing the CBM fused to other bioactive peptides; and the study of the three-dimensional structure of the CBM can be considered.

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