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## Successful production of recombinant buckwheat cysteine-rich aspartic protease in *Escherichia coli*

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**Abstract:** Herein, the expression of recombinant cysteine-rich atypical buckwheat (*Fagopyrum esculentum*) aspartic protease (FeAPL1) in five *Escherichia coli* strains differing in their expression capabilities is presented. It was shown that the expression success depended highly on the choice of FeAPL1 fusion partner. His<sub>6</sub>-FeAPL1 was produced in large quantities as an insoluble protein localized in inclusion bodies. On the other hand, MBP-FeAPL1 was localized in both the cytoplasm and inclusion bodies in BL21 and Rosetta-gami strains. Only purified soluble MBP-FeAPL1 from Rosetta-gami cells showed proteolytic activity at pH 3.0 with BSA as the substrate. The results also indicated that FeAPL1 contained a PRO segment that had to be removed for the enzyme activity to appear. The activity of FeAPL1 produced in the Rosetta-gami strain, which enables disulfide bond formation, indicated the importance of the twelve cysteine residues for correct folding and functionality.

**Keywords:** aspartic protease; His tag; inclusion bodies; MBP; recombinant protein.

### INTRODUCTION

Aspartic proteases (APs) are one of the major classes of proteolytic enzymes and are widely distributed in the whole living world. They are most active at an acidic pH, are specifically inhibited by pepstatin A and contain two aspartic acid residues, which are indispensable for catalytic activity.

The majority of plant APs are distinguished from their non-plant homologues by the presence of the so-called plant-specific insert (PSI), which is removed from most mature plant APs together with the signal peptide and auto-inhibitory PRO segment.<sup>1</sup> Recently a new class of plant APs, often called atypical or AP-like without PSI, has been identified and is represented by six members.<sup>2–7</sup> One of these is FeAPL1, the cDNA of which was isolated from the buck-

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wheat seed cDNA library. Analysis of the polypeptide deduced from the FeAPL1 coding region, predicted an  $M_w$  of 48.6 kDa, four *N*-glycosylation sites and a hydrophobic signal peptide in the *N*-terminal region. Active-site sequence motifs DTG/DSG characteristic for APs as well as twelve Cys residues were also registered.<sup>7</sup>

Interestingly, bioinformatics analysis of the Arabidopsis genome sequence revealed 59 AP-like proteins, providing a new perspective concerning the diversity of AP family members in plants.<sup>8</sup> The biological significance of the existence of two types of APs only in plants is not clear.

Little is known about the biological functions and biochemical properties of the AP-like members. Various functions have been proposed. It was reported that they could be involved in pathogen resistance,<sup>4</sup> in the degradation of rubisco during leaf senescence,<sup>6</sup> in prey digestion<sup>5</sup> or in nucellar cell death.<sup>2,3</sup>

One reason for the lack of data related to the AP-like group is that it is very difficult to obtain sufficiently purified enzyme from plant tissues for detailed characterization. One suitable way is to overexpress the gene in heterologous systems, such as microorganisms or cell cultures of higher organisms (yeast, insects, mammals, *etc.*). The most common host for the production of recombinant APs is *Escherichia coli*. However, of the many attempts, those that gave successful expression often yielded a completely insoluble product that had to be refolded to gain activity. This occurred with OsAsp1 from rice,<sup>3</sup> deleted forms of CND41 from tobacco,<sup>9</sup> cardosin A from *Cynara cardunculus*<sup>10</sup> and some non-plant APs—candidapepsin from *Candida tropicalis*,<sup>11</sup> bovine prochymosin,<sup>12</sup> porcine pepsin<sup>13</sup> and human procathepsin D.<sup>14</sup> The successful production of plant APs in higher organisms was reported for cyprosin and phytepsin in *Pichia pastoris*<sup>15</sup> and insect cells,<sup>16</sup> respectively.

The attractiveness of *E. coli* as an expression system lies in its ability to grow rapidly and at high density on inexpensive substrates, its well characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains.<sup>17</sup> The most important factors that largely affect efficient recombinant protein expression are: a) a strong and tightly regulated promoter (isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible promoters are mostly used); b) *E. coli* strains deficient in the most harmful proteases and/or thioredoxin and glutathione reductases; c) codon usage difference between the *E. coli* strain and the overexpressed gene; d) solubility of the recombinant protein, which depends on the protein expression rate, presence of disulfide bonds, hydrophobicity and choice of fusion partner (tags).<sup>18</sup> The most commonly used tags are polyhistidine (His tag) and glutathione *S*-transferase (GST tag), but tags such as thioredoxin, MBP (Maltose Binding Protein), NusA (transcription termination anti-terminator factor)<sup>19</sup> or SUMO (small ubiquitin-like modifier)<sup>20</sup> have been shown to be more effective as solubility enhancers of fusion partners. For inso-

luble proteins, it is possible to solubilize aggregates and refold proteins either by dilution, dialysis or on-column refolding methods.<sup>21,22</sup> However, optimization of the refolding procedure for a given protein requires time-consuming efforts and is not always conducive to high product yields.

Herein, the production of recombinant buckwheat aspartic protease-like (rFeAPL1) in several *E. coli* strains with two different tags, His<sub>6</sub> and MBP, and an analysis of their efficiency and “stumbling blocks” are reported.

#### EXPERIMENTAL

##### *E. coli* strains and expression vectors

Expression strains: M15(pREP4) (Qiagen), BL21(DE3)pLysS (Promega) BL21-Codon-Plus (DE3)-RIL (Stratagene), BL21-CodonPlus (DE3)-RP (Stratagene), Rosetta-gami (Novagen).

Expression vectors: pQE32 (Qiagen), pMAL-c2X (New England Biolabs).

LB medium: 1 % (w/v) trypton; 1 % (w/v) NaCl; 0.5 % (w/v) yeast extract, pH 7.5,<sup>23</sup> with or without 0.2 % glucose.

LA plates – LB medium containing 1.5 % (w/v) of agar.

Competent *E. coli* cells M15, BL21 and Rosetta-gami were prepared and heat shock transformed according to the manufacturer’s instructions.

##### Preparation of expression constructs

Expression vectors pQE32 and pMAL-c2X were linearized by double digestion using *Sma*I/*Hind*III and *Xmn*I/*Hind*III restriction enzymes (Fermentas), respectively.

The coding sequence of the FeAPL1 gene was amplified using P6:

(5'-atgccggggccacatttccttg-3') and

P5 (5'-gtcaagcttaattttggatcgatcgatcacattgttg-3') primers,

which contain *Sma*I and *Hind*III restriction sites on the 5' ends, respectively. The template was FeAPL1 clone (AY536047).<sup>7</sup>

The polymerase chain reaction (PCR) was cycled 5 times for 30 s at 94 °C, 30 s at 60 °C and 90 s at 68 °C and then 20 times for 30 s at 94 °C and 2 min at 70 °C. Amplification products were cloned in pGEM-T Easy vector (Promega), excised by *Sma*I and *Hind*III restriction enzymes, gel extracted and subcloned into opened pQE32 and pMAL-c2X expression vectors.

##### Recombinant protein production

Recombinant clones containing FeAPL1 cDNA sequence, cloned in expression vectors pQE32 and pMAL-c2X, were used for the transformation of competent M15, BL21(DE3)-pLysS, BL21-CodonPlus (DE3)-RIL, BL21-CodonPlus (DE3)-RP and Rosetta-gami strains.

##### Rapid screening of small expression cultures

Bacterial cultures (2 ml) were grown overnight at 37 °C in LB medium containing ampicillin 100 µg/ml, kanamycin 25 µg/ml in the case of M15 cells, ampicillin 100 µg/ml; chloramphenicol 34.5 µg/ml for all BL21 strains and ampicillin 100 µg/ml, kanamycin 15 µg/ml, tetracycline 12.5 µg/ml and chloramphenicol 34.5 µg/ml in the case of the Rosetta-gami strain.

A 1.5 ml aliquot of prewarmed LB medium (including antibiotics) or LB medium (including antibiotics) containing 0.2 % glucose for expression of FeAPL1 from the pMALc2X vector, was inoculated with 500 µl of the overnight cultures and grown at 37 °C for 30 min with vigorous shaking (180 rpm, Lab-Therm shaker). Expression of recombinant protein was induced by adding IPTG at a final concentration of 1mM and the cultures were grown at 37 °C for 3 h.

The induced cultures (1 ml) were centrifuged at 14000 rpm (Eppendorf centrifuge, 5417R) for 1 min. The pellets were resuspended in 100 µl of Buffer B (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea, pH 8.0). The lysates (10 µl) were analyzed by SDS-PAGE.

Clones that expressed recombinant protein were subjected to determination of protein solubility. One ml of the induced cultures was centrifuged and the pellets were resuspended in the appropriate buffer. Lysis buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 10 mM imidazole, pH 8.0) was used when the expression was from the pQE32 vector and Column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA) for expression from the pMAL-c2X vector. The lysates were sonicated three times for 10 s on ice (Sonipenio 150, MSE (UK) Ltd.) and centrifuged at 14000 rpm (Eppendorf centrifuge, 5417R) for 15 min at 4 °C. The pellets were resuspended in 100 µl of buffer B. The supernatant (20 µl) and pellet (10 µl) were analyzed by SDS-PAGE.

*Large scale production: determination of optimal conditions for recombinant protein production*

The clones with the highest expression of recombinant protein were selected for large scale production. A suitable LB medium (500 to 1000 ml) containing antibiotics was inoculated 1:50 with the overnight culture and grown at 37 °C with vigorous shaking until an *OD*<sub>600</sub> of 0.5 was attained. Expression was induced by adding IPTG to a final concentration of 0.10, 0.30, 0.50 or 1.0 mM.

The cultures were incubated at different temperatures (16, 25, 30 or 37 °C) for 15 min, 30 min, 1, 2 or 3 h. The cells were harvested by centrifugation at 4000 rpm at room temperature for 20 min and resuspended in Lysis buffer or its modifications (300 or 1 mM NaCl, with or without 0.2 % Triton X-100 or Tween 20, 10 % or 50 % of glycerol and 10 mM 2-mercaptoethanol) or Column buffer at 2–5 ml of buffer per gram wet weight of cells. Lysozyme (1 mg/ml) was added and the lysate incubated on ice for 30 min. After sonication three times for 10 s on ice, the lysate was centrifuged at 10000 rpm for 20 min at 4 °C. Both the supernatant and pellet were analyzed by SDS-PAGE.

The supernatants were used for further recombinant protein purification under native conditions and pellets for purification under denaturing conditions and further refolding of FeAPL1.

*Purification under native conditions*

The cleared lysate (supernatant) was mixed with 200–1000 µl of prewashed 50 % Ni-NTA slurry (expression from pQE32) or amylose resin (expression from pMALc2X) and gently shaken at 4 °C for 1 h or overnight. The resin was collected by centrifugation for 30 s at 1000 rpm. The resins were washed 2 to 4 times with 5–10 volumes of Wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, pH 8.0) for 30 min at 4 °C. The protein was eluted in 200–1000 µl of Elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 250 mM imidazole, pH 8.0) (expression from pQE32) or Column buffer containing 10 mM maltose (expression from pMAL-c2X) and analyzed by SDS-PAGE and immunoblot.

*Purification under denaturing conditions*

The pellet resuspended in Buffer B was mixed with 200–1000 µl of prewashed 50 % Ni-NTA slurry by gently shaking at room temperature for 1 h. After collection, the resin was washed once in 5 ml of Buffer B and 2 to 3 times in 5–10 ml Buffer C for 30 min each time. The recombinant FeAPL1 was eluted in 1 to 2 ml of Elution buffer and analyzed by SDS-PAGE and immunoblot.

### Immunoblot analysis

The antibodies used for immunodetection were mouse Anti-His HRP monoclonal antibodies (Qiagen) and Anti-MBP rabbit antiserum (New England Biolabs).

For immunodetection, rFeAPL1 was electrophoresed and transferred to a PVDF membrane (Millipore) in a Fastblot B43 transfer system (Biometra) according to the manufacturer's instructions. After transfer, all immunodetection steps were performed at room temperature. For detection with Anti-His HRP antibodies, the membranes were washed twice for 10 min with TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated in blocking buffer (0.5 % Blocking reagent in 1X Blocking reagent Buffer, Qiagen) for 1 h. After two washings in TBS-Tween/Triton (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05 % Tween-20, 0.2 % Triton X-100) and one in TBS buffer, the membranes were incubated with Anti-His HRP Conjugate solution (1:2000 dilution) in blocking buffer for 1 h. The membranes were washed twice in TBS-Tween/Triton buffer and once in TBS for 10 min each time.

For detection with Anti-MBP rabbit antiserum, the membranes were washed in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1 % Tween-20) and incubated in Blocking buffer (5 % non-fat dry milk in TBST buffer) overnight. The membranes were incubated with Anti-MBP antiserum in 1:10000 dilution in TBST buffer for 1 h. After washing with TBST buffer three times for 5 min each, the membranes were incubated with anti-rabbit IgG peroxidase conjugate (Sigma) (1:10000 dilution) in TBST buffer.

In both cases, chemiluminescence was detected by an ECL-Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer's recommendations. The membranes were covered with mixed reagent A and B (40:1) for 5 min at RT and exposed to X-ray film for 3 to 10 s.

### Protease digestion

Recombinant His<sub>6</sub>-FeAPL1 expressed in M15 cells, or MBP-FeAPL1 produced in BL21 (DE3)pLysS or Rosetta-gami (3 to 20 µg) was mixed with 20 µg of BSA, casein or hemoglobin in 100 µl of suitable buffer and incubated at 37 °C overnight. Samples were analyzed by SDS-PAGE.

Buffers used for digestion: 0.10 M sodium citrate, pH 3.0 and 4.0; 0.10 M sodium acetate, pH 5.0; 0.10 M phosphate, pH 6.0 and 7.0; 0.10 M Tris-HCl, pH 8.0.

## RESULTS

To produce recombinant FeAPL1, its cDNA without the putative signal peptide (1–20 amino acids) was cloned in two expression vectors (pQE32 and pMAL-c2X) and the resulting constructs were introduced into several *Escherichia coli* strains. Cloning into the pQE32 vector resulted in the fusion of six histidines (His tag) in front of FeAPL1 (construct rHis6-FeAPL1). Cloning into the pMAL-c2X vector resulted in the fusion of the MBP tag in front of FeAPL1 (construct rMBP-FeAPL1) (Scheme 1a and 1b).



(a)



(b)

Scheme 1. Scheme of recombinant constructs: rHis6-FeAPL1 (a) and rMBP-FeAPL1 (b). His6 – polyhistidine tag; PRO – auto-inhibitory PRO segment; DTG and DSG – two catalytic sequence motifs; MBP – maltose binding protein tag.

### Expression of *rHis<sub>6</sub>-FeAPL1*

Several bacterial strains (M15(pREP4), BL21(DE3)pLysS, BL21-CodonPlus (DE3)-RIL, BL21-CodonPlus (DE3)-RP and Rosetta-gami) with different properties for expression of recombinant His<sub>6</sub>-FeAPL1 (experimental) were chosen. Eight clones of each strain were screened for recombinant protein production. In every strain, except Rosetta-gami (which did not produce recombinant protein) at least three clones out of eight produced His<sub>6</sub>-FeAPL1 of approximately 46 kDa and no significant difference in quantity was observed. The produced clones were analyzed for protein localization. After induction of expression, cell pellets were resuspended in native Lysis buffer and centrifuged. Both fractions (pellet and supernatant) were analyzed by 12 % SDS-PAGE (Fig. 1a). The recombinant protein was dominantly localized in the pellet-insoluble inclusion bodies in all clones and it was easily visualized by Coomassie staining. On the other hand, recombinant protein in the soluble fraction could only be detected by anti-His antibodies, due to the small amount (Fig. 1b). Approximately 100 µg of soluble protein from 3 l of culture was purified on Ni-NTA resin but the purified protein did not show any activity.

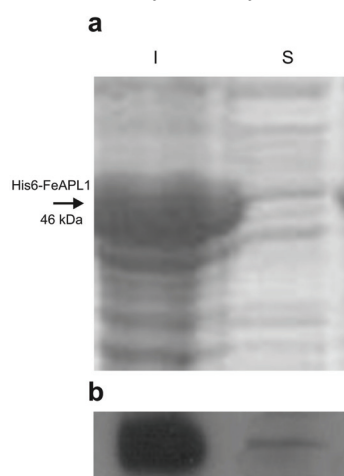


Fig. 1. Expression of rHis<sub>6</sub>-FeAPL1. a) Coomassie brilliant blue G250 staining of 12 % SDS-PAGE of insoluble (I) and soluble (S) fractions of the protein extract of transformed M15 strain. b) Western blot hybridization of the same fractions with Anti-His HRP antibodies.

All efforts to enlarge the soluble fraction, such as altering the induction conditions (temperature, culture  $OD_{600}$ , time of induction, IPTG concentration), changing the Lysis buffer content, or conditions for binding and elution of protein from NI-NTA resin did not give positive results.

### Expression of *rMBP-FeAPL1*

In order to increase the solubility of FeAPL1, an MBP-FeAPL1 construct in the pMAL-c2X vector was made. BL21(DE3)pLysS and Rosetta-gami strains were selected for the expression of this construct. A fusion protein of approxima-

tely 90 kDa was localized in both the insoluble (inclusion bodies) and soluble fractions, with more protein in the inclusion bodies. The recombinant protein from both fractions could be visualized either by Coomassie brilliant blue G250 staining (Fig. 2) or anti-MBP antibody (data not shown). Approximately 1 mg of soluble protein could be purified on amylose resin from one liter of induced bacterial culture.

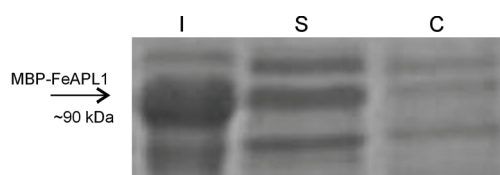


Fig. 2. Expression of rMBP-FeAPL1. Coomassie staining of 12 % SDS-PAGE of insoluble (I) and soluble (S) fractions of protein extract of transformed Rosetta-gami strain and crude protein extract of the untransformed cells (C).

#### *Enzyme activity of rMBP-FeAPL1*

In order to test if the rMBP-FeAPL1 produced from BL21(DE3)pLysS and Rosetta-gami cells showed proteolytic activity, an increasing amount of recombinant enzyme (3, 10 or 20  $\mu$ g) was mixed with 20  $\mu$ g of BSA, casein or hemoglobin in buffers of different pH (experimental). The products of proteolysis were analyzed by SDS-PAGE. Proteolytical cleavage was observed only with BSA in citrate buffer (pH 3.0) with rMBP-FeAPL1 purified from Rosetta-gami cells. The main product was a band of approximately 50 kDa with several lower bands (Fig. 3a). In the presence of 10  $\mu$ M pepstatin A, an inhibitor of aspartic proteases, proteolysis of MBP-FeAPL1 was not observed (Fig. 3b). Purified supernatants from untransformed BL21/Rosetta-gami cells and BSA alone in suitable buffers were used as control reactions (data not shown).

The same reactions were analyzed for autoproteolytic processing of rMBP-FeAPL1 by immunodetection with anti-MBP antibodies. At lower pH values (3.0 and 4.0), there was a clear decrease of the full-length rMBP-FeAPL1 band ( $\approx$  90 kDa) and the appearance of an approximately 44 kDa band, which could correspond to the fusion protein MBP-PRO segment of FeAPL1. This could be evidence that FeAPL1 possesses a PRO segment that has to be removed from the active enzyme. At higher pH values, this band could not be detected and the amount of full-length protein increased with increasing pH (Fig. 4).

#### DISCUSSION

In order to gain insight into the function of FeAPL1 after analysis of its cDNA,<sup>7</sup> it was necessary to obtain the protein itself. It is usually very difficult to isolate sufficiently purified enzyme from plant tissues for detailed characterization, mainly due to the low amounts and poor stability. Therefore, the production of a recombinant protein was chosen. In general, aspartic proteases are “hard pro-

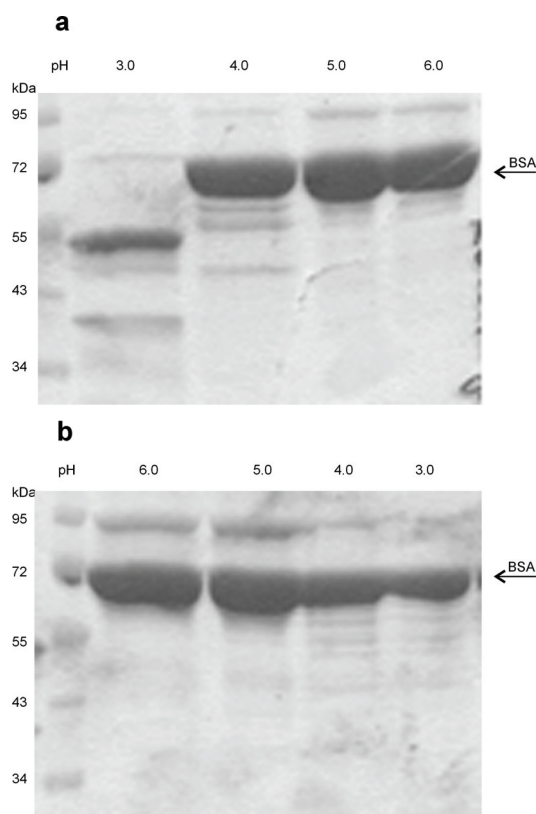


Fig. 3. Enzyme activity of rMBP-FeAPL1. a) Proteolysis of BSA by purified soluble rMBP-FeAPL1 from Rosetta-gami cells in buffers of pH 3.0, 4.0, 5.0 and 6.0 respectively; b) the same reactions in the presence of 10  $\mu$ m pepstatin A. On the left (a) and (b) – PageRuler marker (Fermentas)-bands from top to bottom: 95, 72, 55, 43 and 34 kDa.

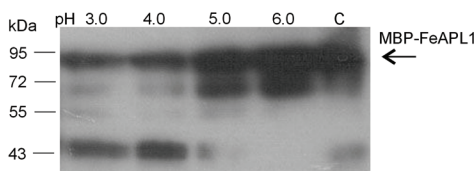


Fig. 4. Autoproteolytic processing of rMBP-FeAPL1. Western blot analysis with Anti-MBP antibodies of the reactions shown in Fig. 3a and purified rMBP-FeAPL1 in Column buffer with 10 mM maltose (C).

teins” for overexpression in heterologous systems. Thus, in *Escherichia coli*, the most frequently used expression system, recombinant APs usually ended up in inclusion bodies. Refolding by different techniques had to be performed in order to make them soluble and active. This justified initial attempts at overexpression in more complex and expensive systems, such as yeast and insect cells. Unfortunately, *Pichia pastoris* did not produce FeAPL1 protein at all, probably due to differences in codon usage, while the protein expressed in insect cells was completely insoluble and inactive. Therefore, it was decided to analyze the *E. coli* system in more detail, being aware that, despite the above-mentioned short-



comings, this system offers many opportunities for variation of all constituents. Production of FeAPL1 was examined in several *E. coli* strains with two different tags – His<sub>6</sub> and MBP.

The difference in codon usage between FeAPL1 and *E. coli* is 27.89 % (Graphical Codon Usage Analyzer, <http://gcu.schoedl.de>). Thus, BL21 RIL and RP strains were chosen in order to overcome possible problems with synthesis of the protein. These strains possess plasmid carrying genes for tRNA for Arg, Ile, Leu and Pro, respectively. Since all strains produced equal amounts of protein, the codon usage difference was shown to have no significance in the efficiency of FeAPL1 expression.

The polyhistidine tag was added to the N terminus of FeAPL1 cDNA without the signal peptide. This tag is poorly immunogenic, small at pH 8.0 and uncharged, and hence does not generally affect compartmentalization or folding of the fusion protein and thus its structure and function.

This system proved very efficient since a large amount of rHis<sub>6</sub>-FeAPL1 (50 mg/l culture) was produced and purified on Ni-NTA resin. However, the crucial problem was the almost complete insolubility of the fusion protein. Factors that might be involved in the formation of inclusion bodies are: a high local concentration of recombinant protein; the reducing environment of the *E. coli* cytoplasm, which prevents disulfide bond formation; the lack of post-translational modification; improper interactions with chaperones and other enzymes involved in folding *in vivo*; intermolecular cross-linking *via* disulfide bonds and other covalent bonds; and increased aggregation of folding intermediates due to their limited solubility. The primary structure of FeAPL1 largely contributes to its insolubility. It contains twelve cysteine residues able to form disulfide bonds. Since all these residues are conserved within the AP-like group, they are certainly important for proper folding into the correct structure and, therefore, functionality of the enzyme. There is no evidence whether all cysteines are included in disulfide bond formation or not. Probably due to the inability of disulfide bond formation, the protein starts to fold improperly, exposing insoluble hydrophobic regions and, together with the factors mentioned above, is prone to aggregation.

For this kind of cysteine-rich protein, it is very challenging to find conditions throughout isolation that will enable the formation of correct intramolecular disulfide bonds instead of intermolecular disulfide bonds leading to protein aggregation. Decreasing the amount of produced protein (decrease of IPTG concentration and/or time of induction), lowering the temperature-increase of protein solubility, and induction of different *E. coli* growth phases did not give any positive results. Therefore, purified His<sub>6</sub>-FeAPL1 from inclusion bodies was subjected to several refolding methods in different buffers. Buffers of different ionic strength, containing detergents such as Triton X-100 or Tween 20, 2-mercaptoethanol as reductant or glutathione oxidized/reduced did not lead to refolding of the FeAPL1.

In order to improve the solubility of FeAPL1, cDNA was inserted downstream from the *malE* gene coding for MBP. MBP has a solubilizing effect on structurally, functionally, chemically and evolutionary diverse proteins, but the exact mechanism is not clear.<sup>24–26</sup>

MBP-FeAPL1 was overexpressed in BL21 and Rosetta-gami cells. Both strains produced a satisfactory amount of soluble fusion protein. The Rosetta-gami strain was chosen because it has a less reducing environment in the cytoplasm (due to disruption of thioredoxin and glutathione reductases), thereby facilitating disulfide bond formation. In addition, it supplies tRNAs for AGG, AGA (arg), AUA (ile), CUA (leu), CCC (pro), GGA (gly). The combination of an MBP-tag and this *E. coli* strain might be crucial for proper folding and correct cysteine positioning for disulfide bond formation. The native structure enables functional proteolytic activity as detected by hydrolysis of BSA at an acidic pH. At an acidic pH, the full-length rMBP-FeAPL1 band ( $\approx 90$  kDa) decreased and a 44 kDa band appeared, that might correspond to the fusion protein MBP-PRO segment of FeAPL1, as indicated with anti-MBP antibodies. This could be evidence that FeAPL1 possesses a PRO segment that has to be removed from the active enzyme, as occurs with many plant APs.<sup>1</sup> This removal was detected at pH 3.0, 4.0, 5.0 but hydrolysis of BSA occurred only at pH 3.0. Broader pH values at which APs are active could be expected.<sup>1,3,5,27</sup> In this study, FeAPL1 activity was detected at pH 3.0, but not at pH 4.0, 5.0 or 6.0. This observation is unusual but one explanation may be that at pH 3.0, when MBP is autocatalytically removed, the protein remains in its native structure and exhibits protease activity. At higher, but still acidic pH values, the protein may possess autocatalytic activity, successfully removing PRO+MBP but after losing MBP, the protein unfolds and loses its activity. Also, the possibility that FeAPL1 is only active in a narrow pH range around pH 3.0 cannot be excluded. Sets of control reactions were set up to confirm that BSA hydrolysis was due to FeAPL1 activity and not to the acidic environment, co-purified bacterial proteases or any other proteases than aspartic. Proteolysis was not detected in reactions with the purified soluble fraction of *Rosetta gami* cells without overexpression of MBP-FeAPL1, or in reactions with BSA in digestion buffers without rMBP-FeAPL1. Finally, when a pepstatin A-specific inhibitor of APs was added, hydrolysis of BSA was absent. The fact that only BSA was hydrolyzed (no casein or hemoglobin) may be interpreted as high substrate specificity of the enzyme, although for such an interpretation, more analyses with different natural and synthetic substrates are required.

One more strategy that could be implemented for FeAPL1 overexpression in *E. coli* is to target it to the periplasm. The beneficial effects achieved through secretion of the gene product include enhanced disulfide bond formation and a considerable reduction in the amount of contaminating proteins in the starting material for purification.

## CONCLUSIONS

Expression of recombinant proteins is a challenging process that demands empirical investigations of all expression system actors, as it is usually impossible to predict the behavior and features of the recombinant protein. Therefore, there are no “hard proteins” if all actors in the expression system are analyzed. These investigations should lead toward obtaining a satisfactory amount of soluble and biologically active protein.

In the case of FeAPL1, the less reducing cytoplasm of the *Rosetta gami* strain in combination with the MBP tag with solubilizing properties on its fusion partner, enabled the production of a satisfactory amount of soluble and, more importantly, proteolytically active enzyme.

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## ИЗВОД

УСПЕШНА ПРОИЗВОДЊА РЕКОМБИНАНТНЕ АСПАРТАТНЕ ПРОТЕИНАЗЕ ХЕЉДЕ БОГАТЕ ЦИСТЕИНОМ У *E. coli*

МИРА Д. МИЛИСАВЉЕВИЋ, ДРАЖЕН Р. ПАПИЋ, ГОРДАНА С. ТИМОТИЈЕВИЋ И ВЕСНА Р. МАКСИМОВИЋ

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У овом раду представљена је експресија рекомбинантне атипичне аспартатне протеиназе хељде (*Fagopyrum esculentum*) богате цистеином, где су тестирана различита експресиона својства пет сојева *E. coli*. Такође је анализиран и утицај фузионих партнера (His<sub>6</sub> и MBP) на ефикасност експресије. У случају His<sub>6</sub>-FeAPL1, добијена је велика количина нерасстворног протеина, смештеног у инклузионим телима. С друге стране, MBP-FeAPL1 је био локализован и у цитоплазми и у инклузионим телима у оба употребљена соја *E. coli* (BL21 и Rosetta-gami). Међутим, само за рекомбинантни протеин произведен у соју Rosetta-gami, доказана је протеолитичка активност на супстрату BSA, при рН 3,0. Резултати су такође указали да FeAPL1 садржи PRO сегмент, чије је одстрањивање неопходно за његову протеолитичку активност. Активност FeAPL1, показана само у соју Rosetta-gami, где је могуће формирање дисулфидних веза, указује на значај 12 цистеина у успостављању правилне структуре која омогућава функционалност ензима.

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