TAS Journal, vol. 3, n. 1, p. 50-63.
ISSN: 2595-1521
MARÇO 2019
adriana.delmonaco@usp.br

Subcloning, expression and purification of Human Hialuronidase-1, variant 8.

Del Monaco ADM*§, Hirata MH§

**Federal University of ABC (UFABC), Brazil. § University of São Paulo (USP), Brazil.*

Abstract. Hyaluronic Acid, HA is a major component of the extracellular matrix of vertebrates. It is a glycosaminoglycan hydrolyzed by enzymes of the hyaluronidase family, involved in the regulation of important biological processes such as angiogenesis and vascular permeability. As interest in the development of a synthesis route for this enzyme, we aim to obtain a plasmid containing the coding sequence of gene variant 8 Hyal-1. To obtain the plasmid insert was planned and two restriction sites for sub-cloning site directed at the 5 'Bam H-1' and 3 'Not-1 in codon sequence of Hyal-1. The insert was sub-cloned into plasmid pET28-a, and transfected for expression in *Escherichia coli* Bl-21. The expression was induced by IPTG in best time of 4 hours and confirmation of protein expression was performed by Western blotting. There was a 45 kDa protein, thus confirming the presence of Hyal-1. Purification was performed on nickel agarose column to obtain a larger amount of the protein, approximately 25μg/L. The route suggested in this study was efficient attainment of Hyal-1 recombinant protein.

Keywords. *Subcloning, Recombinant proteins, Hyaluronic acid, Hialuronidase enzyme.*

Introduction. Among the main components of ECM is hyaluronic acid (HA), which is present in virtually all fluids of upper organs, such as an umbilical cord, without seminal fluid, in the cartilage, among others. In mammals about 50% of all DNA is found in the marks just below the epidermis [1]. HA polymers are synthesized next to cell membrane by the enzyme hyaluronan synthase. The process of translocation of HA polymers, hydrocarbon extracts, through cell membrane has not yet been described [2]. HA is a glycosaminoglycan that can be hydrolyzed in presence of the enzyme Hyaluronoglucosaminidase or Hyaluronidase [3, 4, 5] and is directly related to the diverse functions, among them the adhesion and migration of leukocytes in the connective tissues, mainly T-lymphocytes, which are important mediators of the immune response. Thus, HA may play an important role in T cell function and its interaction with other tissues, especially during the inflammatory process [6].

In addition to composing the ECM, HA is one of compounds that acts as a lubricant in the synovial fluid. In vitro studies have shown that their presence improves chondrocytes' ability to produce extracellular matrix [7]. Thus, HA is directly related to several essential properties of maintaining homeostatic and metabolic balance in animals, acting on the circulation of nutrients, hormones and other chemical signals [8].

Hyaluronidase (Hyal) is an enzyme that was discovered in 1928 by Raynalds et al. But only in 1953 that its function of facilitating the transport of molecules through ECM was discovered by Meyer et al. A few years later the enzyme was named Hyaluronidase [9].

The enzymes in this family hydrolyze the bond between N-acetyl-β-D-glucosamine and D-glucuronate residues on hyaluronic acid polymers, and also catalyze the hydrolysis of 1,4-β-D-glycosidic linkages between N-acetyl- galactosamine and glucuronic acid in chondroitin. It is not yet known why β1,3 binding is not cleaved in the same way [10, 11]. These enzymes are involved in several biological processes, such as increased vascular permeability due to HA fragmentation and consequent decrease in cell-cell adhesion [12].

In humans there are five isoforms of this enzyme: the Hyal-1, -2 and -3 genes are on chromosome 3p21.3 and two other Hyal-4 and PH-20 genes, which in turn are on chromosome 7q31.3 . The Hyal-1 gene encodes a 435 amino acid protein, which has more than 40% homology to PH-20 [13]. While Hyal-2 and PH-20 proteins are linked to cell membrane by glycosylphosphatidyl inositol, which is an anchor protein, Hyal-1 is found entirely soluble [14]. Hyal-2 has a soluble portion and cleaves high molecular weight hyaluronic acid in products of about 20 kDa to about 50 units of disaccharides; Hyal-1 cleaves hyaluronic acid in small disaccharides, with tetrasaccharides as the main product [15].

Hyaluronidase is found in the venom of various species: bees, wasps, scorpions, snakes, fish, mollusks, among other animals. This presence is linked to increased absorption of toxins, potentiating the action of venom [16, 17, 18]. Its relationship with animal poisons has been found since the last decades in various species and continues to this day to be discovered worldwide. Fish of the family Bathracoidae, genus *Thalassophryne*, presented high identity with Hyaluronidase of the *Scorpena* family in their major toxins.

As is the case in accidents with venomous animals, in which the enzyme increases the absorption of the poisons, Hyaluronidase is also known to increase the rate of drug absorption, as it increases the cellular permeability by temporarily decreasing the viscosity of the extracellular medium. There is also a decrease in adhesion between endothelial cells and increased permeability between cells [19].

This property reduces discomfort during the administration of intravenous or intramuscular medications, optimizing the reabsorption of excess fluid and extravasated blood in tissues. It also facilitates the passage of injectable antibiotics, from circulation to the synovial fluid, and administration of subcutaneous saline [20, 21]. In ophthalmologic surgeries HA is used to protect the cornea and Hyaluronidase is used to treat changes in eyeball pressure and to enhance the action of anesthetics [22]. This enzyme is used in plastic and dental surgeries to minimize scar formation [23, 24]. It was also effective in the treatment of vascular extravasations [25], accelerating the reabsorption and degradation of the hematoma quickly.

Polymeric Hyaluronidase microparticles showed great potential as a drug carrier, injectable antibiotics, targeted anti-proliferative agents, corticosteroids, anesthetics, among others [26, 27,

28]. The use in conjunction with controlled drug release polymers, such as contraceptives and insulin, also has important application [29].

Because it is the only one with the property of being completely soluble in plasma, besides being the isoform mostly found in humans, we chose Hyal-1 to target our studies. Such a protein has several variants that result from different types of mRNA processing. Among the variants, the 8 corresponds to the longest transcript, with 1324 bp. The 7 and 8 correspond to the same isoform of the same size, but the one of number 7 has a discrete modification in the 5 'portion as compared to the variant 8. Variants 2, 3 and 5 correspond to smaller transcripts with different structures, with 1160, 692 and 328 bp, respectively, as well as variants 1, 4 and 6, structurally different from the others (NCBI, Gene ID: 3373, 2017). Thus, Hyal-1, variant 8, was the isoform chosen to be subcloned in this project.

Materials and methods.

Synthesis of the oligonucleotides and obtaining the insert:

The sequence corresponding to the cDNA of the Hyal-1 enzyme gene, variant 8, with 1324 bp (Integrated DNA Technologies ®, Iowa USA) was synthesized. The sequences for the restriction sites of the BamH-I and Not-I enzymes chosen, because they were not present in any of the plasmids used nor in the insert, were inserted at the 5 'and 3' ends respectively, as analyzed by the NEBcutter® program , supplied by the restriction enzyme manufacturer (New England BioLabs Inc., Bertle, MA, USA). The recombinant plasmid pIDTsmart-Hyal-1, as shown in Figure 1, was obtained from the synthesized insert cloned into the pIDT-smart® plasmid (Integrated DNA Technologies ®, Iowa USA) for sequential expansion and subcloning, site-directed, in plasmid pET28-a, for expression.

The cDNA was obtained by reverse transcription from total mRNA, performed in vitro. For reaction, we used 1µL Reverse Transcriptase enzyme and 4µl buffer, 1µl of random primers, 1µl dNTP, 2µl DTT and 12µl deionized water and 1µl RNA, for a final volume of 20 µl of reaction. In Verity thermocycler was programmed the first hybridization step 25°C for 10 minutes, then 42°C for 50 minutes for enzyme action, and finally 70°C for 15 minutes for inactivation of enzyme.

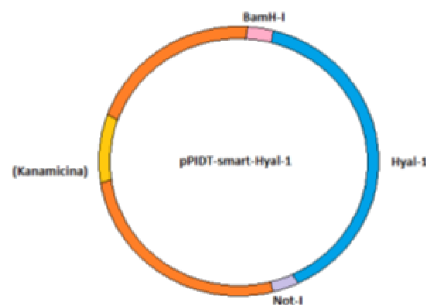

Figure 1: Simplified Scheme of the storage vector with the Hyal-1 insert, pIDTsmart-Hyal-1, with retreatment sites (BamH-I and Not-I), and kanamycin resistance gene.

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Bacterial transformation, plasmid expansion and enzyme restriction:

Escherichia coli bacteria of the Top-10F strain treated with 0.1M calcium chloride (Sambrook & Russel, 2001) were used to become chemo -competent, and thus transformed by thermal shock, for insertion of the pIDTsmart-Hyal vectors -1, which contained the insert.

For each 100µL of competent bacteria were added 10µL of plasmid pIDTsmart-Hyal-1 in a culture tube of 15mL. The mixture was incubated in an ice bath for 30 minutes and then incubated at 42 ° C for 90 seconds, and again cooled for 2 minutes in an ice bath.

To the tube containing the blend was added 900 µL of ice cold SOC medium, liquid Luria Bertani medium (LB) containing 40mM glycerol and 20mM magnesium chloride, and after careful homogenization the culture was placed at 37 ° C for 45 minutes with constant stirring at 50 rpm. Petri dishes containing LB agar and the antibiotic kanamycin at the concentration of 100 µg / mL were seeded 500, 200, 100, 50 and 20 µL of the culture. Plates were incubated at 37 ° C for 16 hours.

Colonies with adequate growth characteristics in this medium were stained in 5mL of liquid LB medium containing kanamycin at 100µg / mL. After incubation at 37 ° C for 16 hours at 160 rpm, the cells were obtained for further purification of the recombinant plasmids using the Plasmid Mini Kit (Qiagen® Strasse, Hilden, Germany) according to the manufacturer's instructions.

Approximately 10µg of each plasmid, pIDTsmart-Hyal-1, were digested with 10U of the BamHI and NotI enzyme (New England BioLabs Inc., Bertle, MA, USA), 5µl of 10X buffer and sterile deionized water in a final volume of 50µL for 4 hours at 37 ° C. This reaction occurred simultaneously, as directed by the manufacturer, since the two enzymes chosen use the same buffer allowing the enzymatic restriction of this form with efficiency close to 100%. Restriction fragments were analyzed by 0.8% agarose gel electrophoresis in 0.5X TBE; and fragments of interest, approximately 1325 bp in size, were purified from the gel using the Illustra GFX ™ PCR DNA and Gel Band kit (GE Helthcare®, Buckinghamshire, England). Prolonged binding with the pET-28-a expression plasmid, also previously cleaved with the same restriction enzymes, was continued according to the same methodology described in this item.

Subcloning into expression vectors:

The subcloning into the expression vectors pET-28-a, which is under the control of the T7 promoter (Merck KGaA®, Darmstadt, Germany), and still contains the polyhedrin tail tail to facilitate purification.

The products of the purified restriction reactions corresponding to the HyaI-1 gene fragment were ligated to the vector pET28a, using insert and vectors in the molar ratio of 3: 1, Ligase buffer containing ATP (1X), 1U of the enzyme T4 DNA Ligase (Invitrogen®, Carlsbad, CA, USA), and deionized water sterile solution in a final volume of 20µL. The binding reaction was incubated for 12 hours at room temperature.

The products of this linkage, pET-28-a-Hyal-1, according to Figure 2, were used to transform competent 0.1M calcium chloride-treated *Escherichia coli* BL-21 cells (Sambrook & Russel, 2001). The bacterial transformation was performed according to the methodology described.

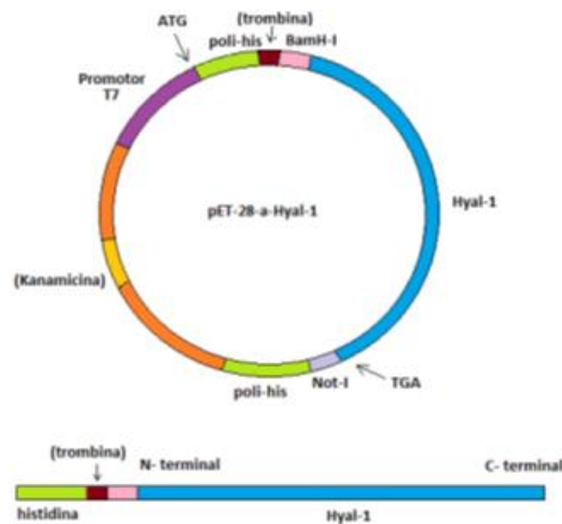

Figure 2: Simplified vector of the expression vector with the Hyal-1 insert, pET-28-a-Hyal-1; with histidine tail (poly-his), start (ATG) and end (TGA) codons, retreat sites (BamH-I and Not-I), and kanamycin resistance gene.

Identification of clones with Hyal-1 sequence:

For identification of the clones containing the sequence of the Hyal-1 transcript gene, fragment amplification was performed by PCR. For this process we used 0.2 µL of the enzyme Platinum Taq® DNA polymerase and 5 µL of buffer (Invitrogen, Carlsbad, USA), 200 µM of each dNTP (GE Healthcare, Buckinghamshire, England), 1 µL of sample, at a concentration of approximately 300 ng / µL, as well as 200 nM of each primer oligonucleotide (Integrated DNA Technologies ®, Iowa USA), as shown in Table 1, in Verity Thermocycler (Applied Biosystem, Foster City, CA).

The program used part of 5 minutes at 5 C for one cycle; follows with 40 cycles of 95 C for 1 minute for denaturation; 3 C for 1 minute for hybridization; 72 C for 1 minute and 30 seconds for the extension step and, finally, a final cycle of 72ºC for 10 minutes for the final extension of the fragment.

DNA Sequencing:

The previously selected pIDTsmart-Hyal-1 plasmids were subjected to DNA sequencing for confirmation of the clones containing the fragments of interest. This step was performed at the Center for Human Genome Studies (Institute of Biosciences, USP, São Paulo, Brazil) using the ABI 3730 DNA Analyzer (AppliedBiosystems®, Foster City, CA, USA) and the Big Dye Terminator v3.1 kit Cycle Sequencing Kit according to the manufacturer's instructions.

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MARÇO 2019
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The sequences were analyzed for confirmation of cloning. First, at the Institute of Biosciences using Sequencing Analysis software 5.3.1, and later in the laboratory, using the BioEdit Sequence Alignment Editor® alignment test.

Determination of the best conditions for Hyal-1 expression:

Plasmid colonies containing the selected plasmids, pET-28-a-Hyal-1, were spiked in 5mL of kanamycin-containing liquid LB medium at 100µg / ml and incubated at 37 ° C, 160rpm for up to 12 hours.

After this period, a new peal was made in 10mL of LB medium in 15mL polypropylene tubes with kanamycin at the 1: 100 dilution, which were incubated at 37ºC, 160rpm, until the logarithmic growth phase, with an optical density between DO600 = 0.5. At that time, expression of the recombinant protein was induced using IPTG (isopropyl-β-D-thiogalactoside) gradient for standardization at concentrations of 0.1mM to 10mM, and the cells again incubated for an additional period of 3-5 hours at 37Â ° C, 160rpm.

An 8 hour incubation at room temperature, 150rpm, was also performed to test expression at lower metabolic rate. At 0, 1, 2, 3 and 4 hours of induction an aliquot of the sample was withdrawn for expression analysis.

Aliquots of 2mL of culture were centrifuged at 14000rpm for 1 minute at 4Â ° C, the pellet resuspended in 6Âµl of the sample buffer and incubated at 100Â ° C for 10 minutes. The samples were then analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) of 12% concentration.

After determining the best condition, a colony of *Escherichia coli* containing the recombinant construct pET-28-a-Hyal-1 was inoculated into 50 ml of liquid LB medium containing 100 µg / ml of kanamycin at 37 ° C, 160rpm for 12 hours . The next day a 2.5µL peal in 250mL of fresh LB medium was made using 50mL overnight culture, same 1: 100 dilution, with kanamycin (100 µg / mL) and the culture was incubated at 37 ° C , 160rpm, with agitation until the log phase of growth (DO600 = 0.5).

At this time, the induction of expression was performed using 1mM IPTG. At the best induction time determined in the previous step, with the highest amount of protein expressed, the cells were recovered by centrifugation, resuspended in PBS buffer and lysed with lysozyme (1 mg / ml). A total of 8 bottles with 250 mL of culture were made, totaling 4 liters, for purification.

Confirmation of expression:

The methodology used to confirm protein expression was Western blotting. Clones that possibly expressed the protein were cultured as described previously in steps 3.4 and 3.5, under the best conditions found. 1mL of culture was centrifuged and the pellet resuspended in 3µL of run buffer (sodium dodecyl sulfate and beta-mercaptanol). This aliquot was separated on 12% SDS-PAGE. and transferred to a nitrocellulose membrane for 90 minutes at 100 V.

After semi-dry transfer the membrane was blocked for 90 minutes in blocking solution (2.5% BSA, 5% Milk powder, 30mL PBS) under constant stirring at room temperature. After this step, the membrane was incubated with 1mL of anti-histidine primary antibodies for 10 minutes, 1: 2000

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concentration, in the Snap-ID® equipment (Millipore, USA) after vacuum washing with 45 mL of PBS buffer (Sodium chloride, monobasic sodium phosphate and dibasic sodium phosphate), incubated with 1 ml of peroxidase-labeled conjugated secondary antibodies (anti-mouse IgG), concentration 1: 1000, and a second vacuum wash under the same conditions described above. Finally, the membrane was incubated for 2 minutes with the developing solution (Western blotting kit Thermo Scientific®, USA) and the chemiluminescence was read, the image acquired by the DNR MF Chemi-Bis (DNR Bio-ImagingSytems®, USA) .

Protein purification:

Purification of the proteins expressed by the bacteria transformed with the pET-28-a-Hyal-1 vector was performed using Nickel-agarose column. A total of four liters of culture, initial, were centrifuged and resuspended in lysis buffer (Tris-HCL pH8 1M, EDTA, 0.5M, 1M PMSF, NaCl, 5M and Lysozyme), sonicated for 10 minutes and centrifuged again at 10,000 rpm, 45 minutes at 4 for purification. The fractions were analyzed on 12% SDS-PAGE gel, and Western blotting, to confirm which fraction had the protein of interest, in the same way described in item 3.7.

After this determination, the pellet, fraction containing the proteins in inclusion bodies, was washed 4 times in buffer (Tris-HCl pH8 1M, 0.5% CHAPSO) centrifuged at 14000rpm for 10 minutes, the supernatants were analyzed on SDS- PAGE 12% and did not contain the protein. The remaining pellet was resuspended in buffer (Tris-HCl pH8 1M, 5M NaCl, 6M Urea, 10% Glycerol), incubated at 37 ° C for 2 hours to scavenge inclusion bodies. After incubation a further centrifugation was carried out at 14000rpm for 30 minutes at 4 ° C, finally, supernatant and pelleted were also analyzed on 12% SDS-PAGE. The fraction containing the protein, which comprises the inclusion bodies which were lysed by 6M urea, present in the buffer (Tris-HCl pH8, 1M, 5M NaCl, 6M Urea, 10% Glycerol) was purified on a nickel-agarose column . During this step the column was washed first with 10mL distilled water, 10mL equilibration buffer (20mM Tris-HCl, 5M NaCl, 5mM Imidazole, 6M urea and 10% Glycerol), sample containing the protein is passed twice, and after this step the 10mL wash buffer (20mM Tris-HCl, 5M NaCl, 20mM Imidazole, 6M urea and 10% Glycerol) is passed. Next, we pass the reuptake buffer solutions (20mM Tris-HCl, 5M NaCl, 20mM Imidazole, 10% glycerol, 0.1% Triton, 0.5mM GSSH, 0.5mM GSH), gradient 6M, 4M, 2M, 1M, 0.5M and 0M urea, 5mL each.

Finally, 5mL elution buffer (3.5mL renaturation buffer and 1.5mL Imidazole) is added, reprocessed on 12% SDS-PAGE and show the presence of the pure protein. After this step, the samples were dialyzed in solution (50mM Tris-HCl pH8, 50mM NaCl, 50% Glycerol, 0.1 mM DTT, 0.1mM PMSF and 500mL distilled water) with stirring at 10 ° C and quantified Pierce BCA Thermo Scientific®, USA).

Results and discussion.

Synthesis of the oligonucleotides and obtaining the insert:

The chemical synthesis of the sequence of interest containing the sequence encoding the mRNA of the Hyal-1 gene, variant 8, inserted into the plasmid, pIDTSmart, was confirmed by sequencing.

TAS Journal, vol. 3, n. 1, p. 50-63.
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MARÇO 2019
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The peak intensity in the absorbance plot by found base and comparison with the standard sequence, initially by the Institute of Biosciences using Sequencing Analysis software 5.3.1; and later in the laboratory, using the BioEdit Sequence Alignment Editor® alignment test, showed a satisfactory sequencing quality and absence of possible mutations in the insert.

Bacterial transformation, plasmid expansion and enzyme restriction:

The pUCIDT-smart ® plasmids were expanded to an amount sufficient to proceed with the enzyme restriction reaction to obtain the insert, totaling approximately 200µl of plasmid, at a concentration of 400ng / µl. The best insert-plasmid ratio ratio was 3: 1. The enzymatic restriction reaction of pUCIDT-smart® plasmids, with the insert, and subcloning into the pET-28a® vector, according to the methodology described in 3.2, generated the fragments shown in Figure 3, which were extracted and purified from the gel as described in the same item.

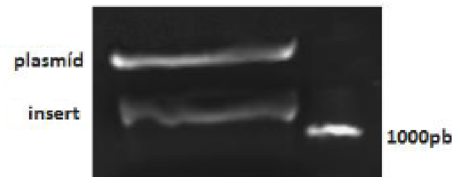

Figure 3: Photograph of the agarose gel 8% of electrophoresis showing the fragments of plasmid pIDTSmart and insert of the Hyal-1 gene after enzymatic restriction with BamH-1 and Not-1.

Subcloning of expression vectors and identification of clones with sequence Hyal-1:

The inserts were then subcloned into the pET-28a® expression vectors, and named pET-28-a-Hyal-1. After the new bacterial transformation and the analysis of recombinant clones, which were performed according to the methodology described in 3.2 and 3.3, we obtained approximately 1300bp fragment size identified in nine different clones in bacteria transformed with pET-28-a-Hyal-1, which indicates the presence of the insert. As shown in Figure 4.

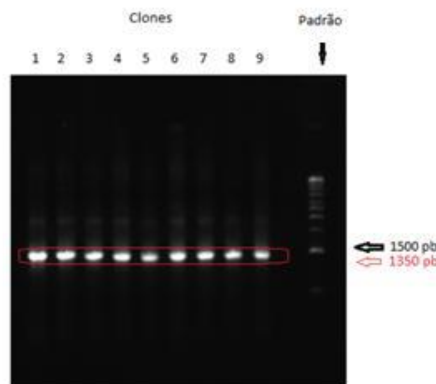

Figure 4: Photograph of the electrophoresis gel in 0.8% agarose, showing fragments of the PCR products corresponding to the Hyal-1 gene, variant 8.

The cloning confirmation in the pET28-a vector by sequencing was performed to certify the insertion at the correct position in the selected clones. Of the five clones analyzed, all presented the expected results of sequencing.

Determination of the best conditions for Hyal-1 expression:

The expression was performed according to the described methodology, but the gel electrophoresis analysis 12% SDS-PAGE showed no significant difference in the samples containing the total of 1mL bacterial culture, lysed by thermal shock, before and after induction with IPTG, according to Figure 5, so a new study of IPTG concentration gradient and induction temperature decrease and time increase to 8 hours was performed to test the expression of this protein in metabolic rate of the smallest bacterium, and in different concentrations of IPTG.

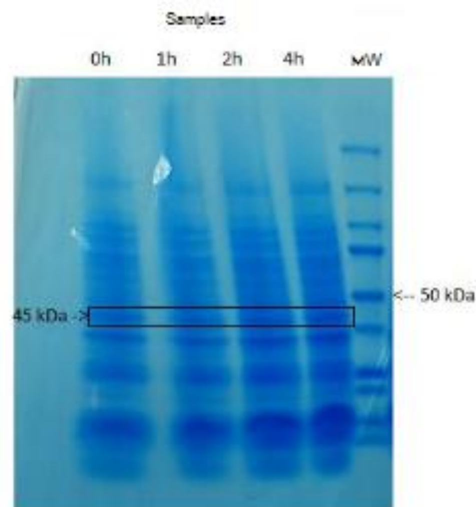

Figure 5: Photograph of electrophoresis in 12% polyacrylamide, highlighting the 45 kDa size bands of the samples at 0, 1, 2 and 4 hours after expression induction.

Expression confirmation:

We proceeded with Western blotting to confirm protein detection in these new tests, since electrophoresis was not efficiently discriminatory. Clones in which expression was performed for 8 hours at 38 ° C with a concentration of 1mM IPTG showed the best results in this experiment. We confirmed that the induction of expression at room temperature for 8 hours was not significant, as well as concentrations below or greater than 1mM IPTG. As shown in Figures 6 and 7, below.

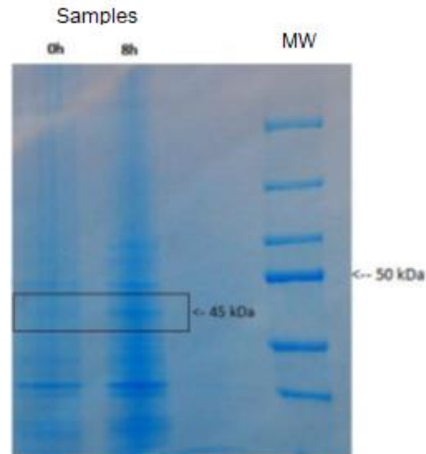

Figure 6: Photograph of 12% polyacrylamide electrophoresis gel, highlighting the 45 kDa size bands of the samples at times 0 and 8 hours after expression induction at room temperature.

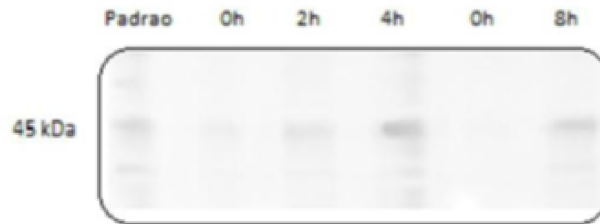

Figure 7: Photograph of Western blotting membrane, highlighted the bands with 45 kDa size of samples at times 0, 2 and 4 hours after induction of expression at 38 ° C and 0 and 8 hours after induction at room temperature.

Protein purification:

We performed the purification of samples in a nickel-agarose column, according to the methodology item 3.8. Thus, we could confirm the expression of Hyal-1 thus standardizing its best found conditions: Concentration of IPTG of 1mM, temperature of 38ºC, stirring constant 160 rpm for 4 hours, after induction and obtaining the insoluble fraction, with denaturation of the inclusion bodies, according to Figure 8.

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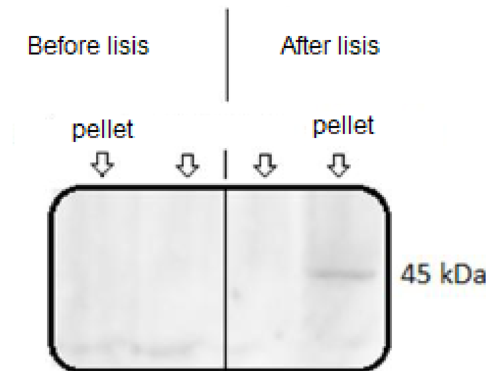

Figure 8: Photograph of the Western blotting membrane of the samples before and after the ultrasonic lysis, respectively, highlighting the band with size of 45 kDa present only in the sample of the sedimented after lysis, which contains the insoluble fraction, with the bodies of inclusion.

During the purification step the washes of the nickel-agarose column were carried out with urea solutions in a gradient of 4, 2, 1, 0.5 and 0M, as described in item 3.8. for protein renaturation. Therefore, the elution fractions already contain the protein in its renaturated form.

The elution fractions at the end of the purification showed the electrophoresis profile according to Figure 9A, and Western blotting Figure 9B. We can perceive the presence of a band with 45kDa in the electrophoresis of elution fraction 1, but in the polyacrylamide gel was not However, in Western blotting, which has a higher sensitivity and specificity, it was already possible to observe the bands in the two elution fractions, with concentration 25µg/l.

Figure 9: A- Photograph of 12% polyacrylamide electrophoresis gel, highlighting the 45 kDa size bands of the samples from elution fractions 1 and 2 of the samples and the urea gradient passages during purification by liquid chromatography on a nickel column. B- Photograph of Western blotting membrane of samples after purification on liquid chromatography on a nickel-agarose column, samples 1 and 2 correspond to the elution fractions, and samples 3 and 4 are the column wash fractions, showing that no recognition occurred in these samples. Highlighted the bands with size of 45 kDa.

Conclusion.

The flanking region of Hyal-1, variant 8, produced by chemical synthesis route was feasible in the present study.

Subcloning, site-directed, into pET-28-a-Hyal-1 expression plasmid added to the polyhistidine tail proved to be a good strategy for purification of the recombinant Hyal-1 protein.

Expression of Hyal-1 in *Escherichia coli* can be confirmed only by Western blotting. Further purification of the recombinant protein using nickel-agarose column can be confirmed by electrophoresis in 12% polyacrylamide.

The yield corresponding to 25 µg per liter of initial crop suggests production viability for future investments.

Acknowledgments. We thank CAPES for funding.

Disclosure. The authors report no conflicts of interest in this work.

TAS Journal, vol. 3, n. 1, p. 50-63.
ISSN: 2595-1521
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