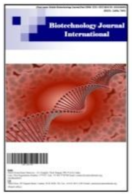

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Recombinant Co-Expression of Collagen A1 (I) Fragment with the Prolyl 4-Hydroxylases (P4H) Subunits in *Komagataella phaffii*

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Authors' contributions

This work was carried out in collaboration between both authors. Author ZK designed the study and managed the literature searches. Author EA performed the laboratory analysis under the strict supervision of author ZK and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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ABSTRACT

Recombinant collagen and collagen-like products are increasingly replacing animal-sourced collagen that is difficult to produce in safe and standard quality. In this study to produce hydroxylated collagen, a 400 base pair collagen fragment of the bovine COL1A1 gene was co-expressed with prolyl-4-hydroxylase subunit α (P4Hα) and prolyl-4-hydroxylase subunit β(P4Hβ) encoding the P4H enzyme in *Komagataella phaffii*. For this purpose, each target gene was inserted into the pPICZαA vector and then cloned in *E. coli* DH5α cells. Subsequently, co-expression vectors were constructed using recombinant vectors isolated from positive clones according to the *in vitro* multimer ligation method. All recombinant expression and co-expression vectors were transformed into *K. phaffii* X33 cells by electroporation. The results of reverse transcriptase-polymerase chain reaction (PCR) proved that all target genes were transcribed by recombinant strains. The expression of recombinant proteins was performed for 96 hours by methanol-fed cultivation, and the concentration of the purified proteins from the culture medium was measured by the His-Tag enzyme-linked immunosorbent assay (ELISA) method. The concentrations of rP4Hα and rP4Hβ, and rCol1 proteins expressed individually by recombinant strains were determined to

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be 10.69 µg/L, 10.74 µg/L, and 8.61 µg/L, respectively, while the concentrations of co-expressed rP4Hα/β and rP4Hα/β/rCol1 proteins were 7.82 µg/L and 5.02 µg/L, respectively. These results showed that the target genes were successfully expressed and co-expressed in the recombinant *K. phaffii* cell.

Keywords: Recombination; pPICZαA; collagen; expression; Komagataella phaffii; prolyl 4-hydroxylases.

1. INTRODUCTION

Gelatin is an animal-derived protein that has a unique mechanical function. Gelatin is widely used in many areas, such as in biomedical, pharmaceutical, chemical, food, and cosmetic fields. Gelatin is generally produced by acidic or alkali extraction from collagen [1-4]. But the gelatin sourced from animal tissues has several associated risks, such as pathogenic elements like viruses and prions, as well as allergic reactions [5]. The quality of the final product varies with the animal's breed, age, physiological condition and the production process is often non-standardized [6]. Also, the varying batch-to-batch product quality is a significant problem, especially in biomedical applications [7].

Therefore, there is a need for an alternative gelatin production strategy to overcome the disadvantages associated with the use of animal-derived gelatin. In the last decades, recombinant DNA technology has been getting more and more attention to producing functional proteins [8]. Many studies have reported that recombinant collagen has been successfully expressed in various host organisms, such as transgenic mice, tobacco, silkworm and mammalian cell lines [7]. Besides plants and animal host organisms, microbial hosts such as bacteria and yeast are also very popular because they allow the inexpensive production of recombinant collagen in a short time [9]. Eukaryotic yeast expression systems can successfully produce and secrete biologically active animal-derived proteins. The *K. phaffii* expression system in particular has several advantages, such as post-translational modification, intracellular secretion, and a well-known genetic structure [10].

Natural collagen has a triple helix conformation of α-peptide chains characterized by a repeating Gly-XY sequence motif [11]. In repetitive Gly-XY sequences, X and Y are proline and hydroxyproline respectively. The hydroxylation of the proline residues at the Y position of the procollagen by the prolyl 4-hydroxylase (P4H) enzyme is necessary for the formation of the characteristic molecular conformation of the collagen in the triple helix structure. The human P4H enzyme is a heterogeneous tetramer consisting of 2α and 2β subunits; the α subunits are responsible for catalytic activity, while the β subunits are known to keep the α subunits in a catalytically active conformation [12-15].

Hydroxylation plays an important role in maintaining the thermal stability of collagen. Therefore, in the production of recombinant collagen, both subunits of the P4H enzyme must be produced in the expression system to establish the characteristic molecular conformation [15]. Recombinant gelatin production is based on the expression of collagen gene fragments with specific lengths and composition in a variety of host organisms. It has been shown that recombinant gelatin produced by the expression of collagen fragments between 99 and 101 amino acid lengths can be used instead of animal-derived gelatin in many medical applications [16].

In this study, the aim was to express and co-express the collagen fragment and two subunits of the P4H enzyme in *K. phaffii* host cells to produce recombinant gelatin. For this purpose, the *in vitro* multimer ligation protocol was followed for the co-expression of target genes in the *K. phaffii* host cell, and the recombinant proteins were quantitively analyzed.

2. MATERIALS AND METHODS

2.1 Strains and Vectors, Reagents and Culture Media

In this study, the pPICZαA (Invitrogen, USA) vector was used for cloning and expressing of interesting genes. The vectors, recombined with target genes were cloned in *Escherichia coli* strain DH5 α (Invitrogen, USA). *Komagataella phaffii* strain X-33 (Invitrogen, USA) was used as the host to express the recombinant proteins. The enzymes used during all recombination procedures and the DNA and RNA isolation kits and PCR reagents were purchased from Transgen Biotech (Beijing, China). *E. coli* DH5α and *K. phaffii* X-33 cells were cultured in Luria-Bertani broth (LB) and Yeast Extract Peptone Dextrose Medium with sorbitol (YPDS), respectively. The buffered glycerol-medium yeast extract (BMGY) was used for propagation of *K. phaffii* while induced expression of recombinant proteins was performed in the buffered methanol-medium yeast extract (BMMY).

2.2 Construction of Expression and Co-Expression Vectors

In this study, a 400 bp fragment between 2787 to 3189 on the collagen type I alpha 1 gene (COL1A1), (NCBI Accession number NM_001034039) from *Bos taurus* was targeted. To obtain the collagen gene fragment, named as "Col1" the RNA was isolated from bovine skin using RNA isolation kit and then amplified by reverse transcriptase PCR (RT-PCR) using primers which were specific to the target gene region. The 5'- and 3'- ends of the specific primers contained EcoRI and NotI restriction sites respectively (Table 1). One-step RT-PCR was performed the cycle conditions as follows; reverse transcription at 50°C for 25 min followed the first denaturation at 95 °C for 5 min, and then for 30 cycles of denaturation at 95°C for 10 s, annealing at 63°C for 5 s, and extension at 72 °C for 25 s. The PCR product was restricted by EcoRI and NotI enzymes and then ligated to pPICZaA vector using T4 DNA ligase [17]. The recombinant expression vectors, pPICZαA-P4Hα and pPICZαA-P4Hβ, containing the P4Hα (NCBI Accession number: NM_000917) and P4Hβ genes (NCBI Accession number: NM_000918), respectively, were obtained from Genescript (NJ, USA).

Co-expression vectors were constructed according to *in vitro* multimers protocol described by Cregg et al. [18]. For this, the expression vectors containing any target gene were digested BglII and BamHI to release the expression cassette. Subsequently, the BglII/BamHI restricted expression cassette was ligated into the BamHI linearized expression vector already containing another target gene. In this way, two co-expression vectors which were combined the P4Hα and P4Hβ genes and P4Hα/P4Hβ and Col1 genes were constructed (Fig. 1).

The recombinant and co-expression vectors were transferred into $CaCl_2$-competent *E.coli* DH5α cells by heat shock treatment [19]. *E.coli* DH5α cells were grown in Luria-Bertani agar containing 25 µg/mL zeocin for the propagation of the recombinant vectors. Colonies growing on zeocin-containing agar were considered positive and confirmed by PCR amplification of target gene fragments. PCR products were run on 1.5% agarose gel and the bands with expected size were visualized.

2.3 Transformation of Recombinant Vectors into *K. phaffii* Cells

The recombinant vectors were isolated from an overnight culture of *E.coli* DH5α clones using a commercial plasmid isolation kit. The purified expression vectors were linearized with SacI enzyme and then electroporated (1.500 V/cm) into *K. phaffii* X33 cells [20]. The co-expression vectors containing more than one expression cassette were transformed into *K. phaffii* X33 cells in non-linearized circular form. Positive *K. phaffii* X33 transformants were selected from YPDS agar plates containing zeocin (0.4 - 1mg/mL) after incubation at 30°C for 3-5 days. The presence of the target genes in positive transformants was checked by PCR using specific primers [21]. PCR amplifications were performed by following the reaction conditions: initial denaturation at 95ºC for 10 min, followed denaturation at 95ºC for 20 s; annealing at 50-63ºC for 20 s; extension at 72ºC for 30 s during 35 cycles, and final elongation at 72ºC for 5 min. Amplification products were visualized on 1.5% agarose gel. A total of 5 different recombinant *K. phaffii* X33 strains were obtained, transforming with a different expression and co-expression vectors.

2.4 Controlling of Transcription

The total RNAs isolated from the recombinant *K. phaffii* strains were used as a template for RT-PCR amplification of target genes. The one-step RT-PCR procedure was applied according to; reverse transcription at 45ºC for 30 min followed by initial denaturation at 95ºC for 5 min and 35 cycles of denaturation at 95ºC, 30 s; annealing at 50ºC, 30 s; and an extension at 72ºC, 40 s, and a final elongation step at 72ºC for 5 min. PCR products were controlled by running 2% agarose gel. Amplification products were subjected to sequence analysis for controlling transcription of target genes.

2.5 Recombinant Protein Production Using Shake-flask Cultivation

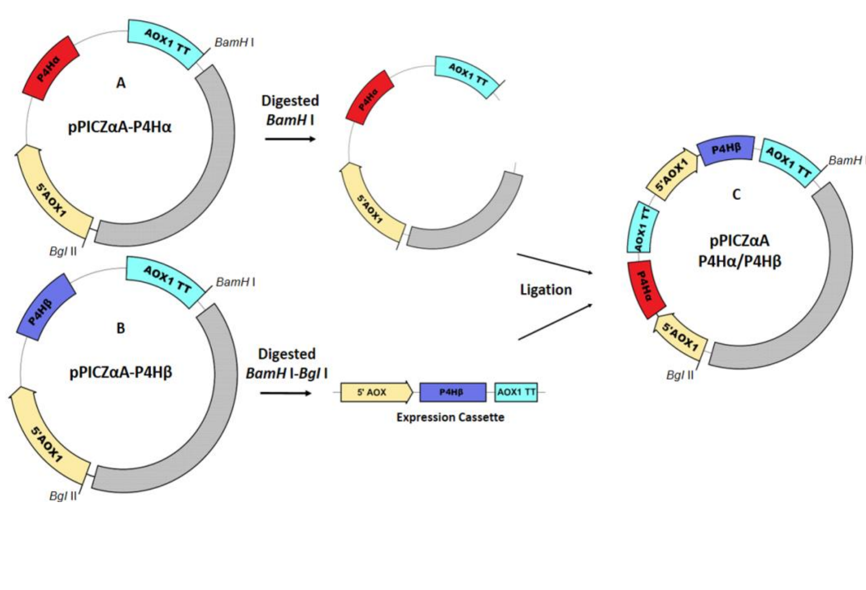
The recombinant strains were subjected to methanol-fed cultivation for recombinant protein production [22]. The selected positive transcripts were grown on YPD agar for 48 hours. The harvested cells were transferred into 4 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4x10-5% biotin, and 1% glycerol) and incubated at 30°C for 18 hours with shaking at 250 rpm. The cells were precipitated by centrifugation at 4000 x g after the OD_{600} value of the culture medium reached the range of 2-4. The cell pellet was transferred into 500 mL flasks containing 20 mL of BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4x10-5% biotin, and 0.5% methanol) medium and then incubated at 30°C with shaking at 225-250 rpm for 96 h. After 0.5% methanol induced incubation stage the culture medium was centrifuged at 12000 x g for 20 min at 4°C and the supernatant was precipitated with ammonium sulfate at a final concentration of 60% (w/v) [23]. The pellet was resuspended in 1 ml PBS buffer and dialyzed overnight in 0.1 mol/L sodium acetate using 30-3 kDa molecular weight cut off (MWCO) dialysis tubing.

2.6 Quantification by Recombinant Protein Using His-Tag ELISA

Quantification of recombinant proteins purified from the culture medium was performed using a commercial His-Tag ELISA kit (Shanghai YL Biotech Co. Ltd., China) according to manufacturer instruction. The calibration curve was plotted using 6 different standard solutions in the concentration range of 150 pg/mL-4800 pg/mL and the linear regression equation was calculated. The recombinant proteins were loaded into wells pre-coated with His-Tag monoclonal antibody and then incubated. After the addition of anti-His-Tag antibody labeled with biotin and streptavidin-conjugated horseradish peroxide (HRP), unbound enzymes were removed by washing. The chromogen solutions A and B were added, respectively, and then incubated for 10 min. After the addition of the stop solution, the absorbance (OD) of each well was measured at 450 nm wavelength. The concentrations of the recombinant proteins were calculated using the linear regression equation of the standard curve.

2.7 SDS-PAGE Analysis of Recombinant Proteins

The recombinant proteins were separated in sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) gel, consisting of a 4% stacking gel and a 10% separating gel. At the end of the electrophoresis performing at 200 volts for 1 hour, resolved proteins were visualized through Coomassie Brilliant Blue staining [24]. The sizes of protein bands were compared with the molecular weight marker (20-200 kDa).



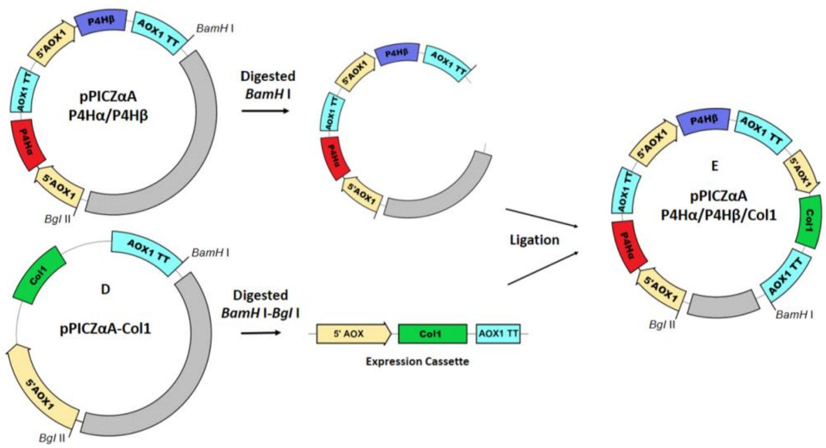


Fig. 1. Construction of expression and co-expression vectors

A: pPICZαA-P4Hα vector, B: pPICZαA-P4Hβ vector, C: pPICZαA-P4Hα/P4Hβ vector, D: pPICZαA-Col1 vector and E: pPICZαA-P4Hα/P4Hβ/Col1 vektor

Table 1. The sequences of the primers used in this study

Primer Name	Sequence 5'→3'
P4Hα-F	AATTATAGGAATTCTGCTTCCCCAGA
P4Hα-R	TTCCAATTCTGACAACGTACAA
P4Hβ-F	ACGTCCTGGTGCGGAAAAG
P4Hβ-R	CAGTTCATCTTTCACAGCCTGAT
Col1-F	C**GAATTC**AGACCAGACTGGCAACAGCT
Col1-R	GAT**GCGGCCGC**AGGAAGACCAGGGAAG

GAATTC: EcoRI restriction site; GCGGCCGC: NotI restriction site

3. RESULTS

3.1 Construction and Control of Recombinant Vectors

In this study, RNA isolated from bovine skin was used as a template to obtain a 400 bp of the collagen gene fragment. The target gene fragment was amplified by RT-PCR using the Col1-F and Col1-R primers and then ligated to pPICZαA vector after cutting with EcoRI and NotI. On the other hand, two custom pPICZαA vectors, integrated with the genes of P4Hα and P4Hβ coding the P4H tetramer were purchased from Genscript. Also, two co-expression vectors; pPICZαA- P4Hα/P4Hβ and pPICZαA-P4Hα/P4Hβ/Col1 were constructed using *in vitro* multimer ligation protocol. All expression and co-expression vectors were transformed into chemical-competent *E. coli* DH5α cells using the heat shock method. Positive clones were selected from LB agar containing zeocin and recombinant vectors were isolated from the overnight bacteria culture in LB medium. The presence of target genes in each expression and co-expression vector was verified by PCR using specific primers. Amplification products were run on a 1.5% agarose gel and the specific bands of P4Hα, P4Hβ, and Col1 fragments in the lengths of 1523 bp 1583bp and 400 bp, respectively were observed. The co-expression vector, pPICZαA-P4Hα/P4Hβ contained the bands of P4Hα and P4Hβ genes in expected length, while all of three bands of target genes were observed in the pPICZαA-P4Hα/P4Hβ/Col1 co-expression vector (Fig. 2).

3.2 Transformation of Recombinant Vectors into *K. phaffii* Cells

Recombinant vectors isolated from positive *E. coli* DH5α strains were transformed into *K. phaffii* X33 cells by electroporation and transformants were selected from YPD agar containing zeocin. The presence of target genes in transformants was checked by PCR and bands in expected lengths were observed in all zeocin-resistant transformants (Fig. 3).

3.3 Controlling of Transcription

To control transcription, total RNAs were isolated from positive *K. phaffii* transformants and target gene fragments were amplified by RT-PCR using specific primer sets. The transcription of the Col1 gene was confirmed by sequence analysis of the amplification products (Fig. 4). When compared to the sequence results with the NCBI database showed 99% similarity to corresponding target genes (Table 2).

3.4 SDS-PAGE Analysis of Recombinant Proteins

At the end of the methanol-induced expression stage, the recombinant proteins produced by *K. phaffii* transformants were purified using ammonium sulfate precipitation. The purified proteins separated in %10'lık Tricine-SDS-PAGE polyacrylamide gel. The bands of rPH4α, rPH4β proteins in approximately 50 kDa molecular weight, and the band of rCol1 protein of 13 kDa were observed after staining of SDS-PAGE gel with coomassie brilliant blue R250 (Fig. 5).

3.5 Quantification of Recombinant Peptides by ELISA

The quantitive detection of recombinant proteins, purified from the culture medium was performed by sandwich enzyme methods using commercial His-Tag ELISA kit. The concentrations of the recombinant proteins were calculated according to the linear regression equation, obtained using the OD values of standard solutions (Table 3). The concentration values of purified recombinant proteins changed 5,02-10,74 (µg/L). The amount of recombinant proteins produced by strains containing a single gene was higher than those produced by recombinant strains transformed with two or three genes.

Table 2. The accession number of genes found in the NCBI database when searched the sequence of target genes transcripted by positive transformants

Target Gene	Accession Number	% Similarity
P4Hα	NM_001017962.2	%100
P4Hβ	NM_000918.4	%99
Col1	NM_001034039.2	%99

Table 3. The concentration of recombinant proteins purified from the culture medium

Recombinant Proteins	Concentration (µg/L)
rP4Hα	10.69
rP4Hβ	10.74
rCol1	8.61
rP4Hα/β	7.82
rP4Hα/β/rCol1	5.02

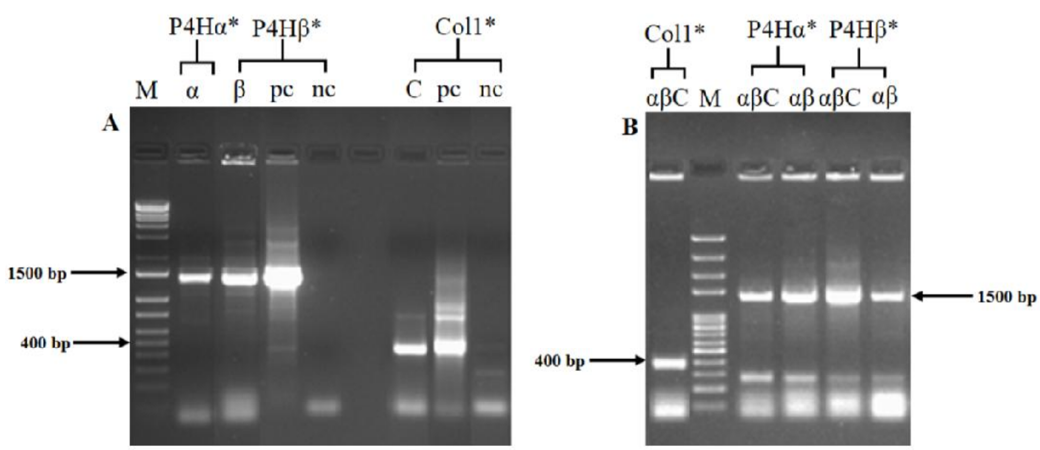


Fig. 2. Agarose gel showing the bands obtained by amplification of target genes cloned in *E. coli* DH5α. A: Amplification products of single target gene transformed into *E. coli DH5α* and B: Amplification products of multiple target genes transformed into *E. coli* DH5α

M: DNA marker; α: P4Hα gene; β: P4Hβ gene; 3: Col1 gene; pc: Positive control. nc: Negative control M: DNA marker; αβ: P4Hα/P4Hβ genes; αβC: P4Hα/P4Hβ/Col1 genes; C: Col1 gene; P4Hα: Amplification of product obtained with P4Hα-F/R primers; P4Hβ*: Amplification of product obtained with P4Hβ-F/R primers; Col1*: Amplification of product obtained with Col1- F/R primers*

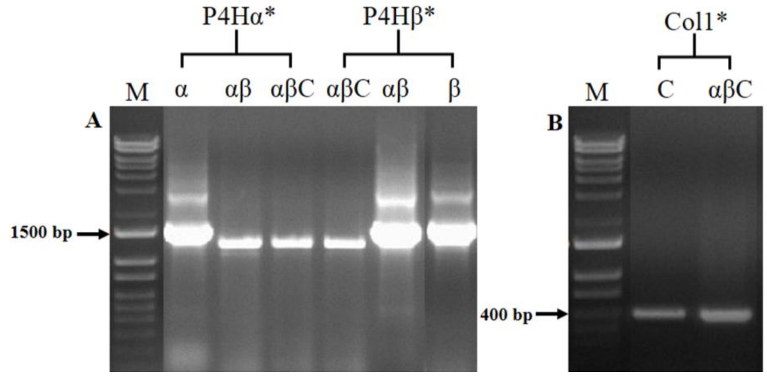


Fig. 3. Agarose gel showing the bands obtained by amplification of target genes transformed into *K. phaffii*

M: DNA marker; α: P4Hα gene; β: P4Hβ gene; αβ: P4Hα/P4Hβ genes; αβC: P4Hα/P4Hβ/Col1 genes; C: Col1 gene; P4Ha: Amplification of product obtained with P4Hα-F/R primers P4Hb*: Amplification of product obtained with P4Hβ-F/R primers Col1*: Amplification of product obtained with Col1- F/R primers*

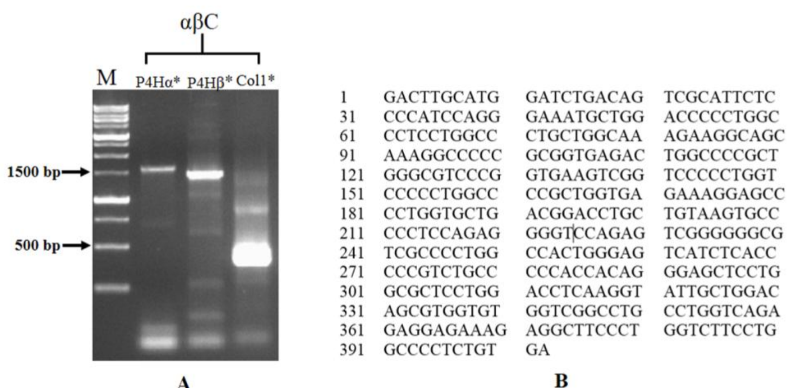


Fig. 4. Agarose gel showing transcription results of the target genes

A; M: DNA marker; αβC: P4Hα/P4Hβ/Col1 P4Ha: Amplification of product obtained with P4Hα-F/R primers P4Hb*: Amplification of product obtained with P4Hβ-F/R primers Col1*: Amplification of product obtained with Col1- F/R primers B; Sequencing result of Col1*

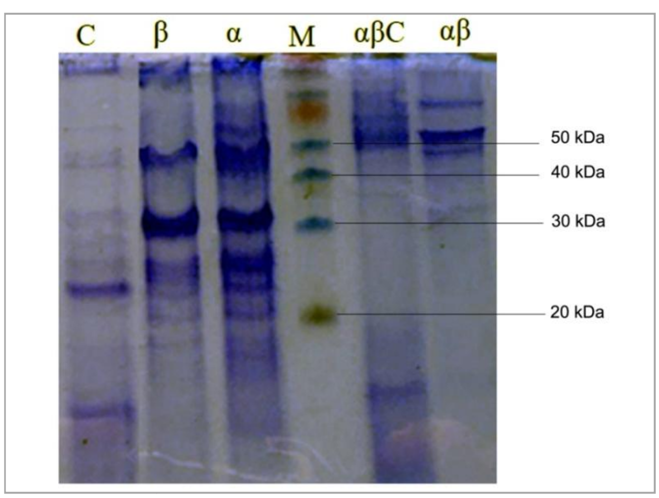


Fig. 5. SDS-PAGE gel showing recombinant proteins

M: Protein standard α: P4Hα, β: P4Hβ, αβ: P4Hα/P4Hβ, αβC: P4Hα/P4Hβ/Col1

4. DISCUSSION

There is an increasing need for the production of gelatin to be used specifically in biomedical and pharmacological applications in a safe way, with desirable textural properties and to a standard quality [25]. Animal-derived gelatin may increase the risk of zoonotic diseases, and it also has other disadvantages such as the lack of a standard and environmentally friendly production method. In recent years, recombinant DNA technology has been considered to be an effective approach in the production of collagen and collagen-like materials to overcome these deficiencies [26,27].

The formation of the triple helix conformation of collagen is associated with the hydroxylation of proline residues in procollagen by P4H enzyme. P4H has an essential role in maintaining the stability of the collagen triple helix structure [28,29]. For this purpose, in this study, to producing recombinant gelatin a 400 bp fragment of the bovine COL1A1 gene, was co-expressed with the α (P4Hα) and β (P4Hβ) subunits of the P4H enzyme.

Therefore, the expression vector, pPICZαA-Col1 including a 400 bp fragment that rich in glycine and proline on the bovine COL1A1 gene was constructed. The pPICZαA-P4Hα, pPICZαA-P4Hβ vectors were obtained from Genscript. Also, the co-expression vectors; pPICZαA-P4Hα/β and pPICZαA-P4Hα/P4Hβ/Col1 were constructed used *in vitro* multimer ligation strategy. All of the expression and co-expression vectors were cloned to *E. coli* DH5α cells. The positive *E. coli* DH5α clones were selected from zeocin containing LB agar and the recombinant vectors were isolated and then transformed into *K. phaffii* X33 strains. In the result, 5 different *K. phaffii* X33 recombinant strains were obtained expressing rP4Hα, rP4Hβ, rCol1, rP4Hα/rP4Hβ, and rP4Hα/rP4Hβ/rCol1 proteins. The methanol-induced protein production was performed and then the proteins purified from the culture medium were quantified.

In this study, after the constructed pPICZαA-Col1 vector was transformed into *K. phaffii* X 33 strain, RNA was isolated from positive transformants, and then transcription was controlled by RT-PCR. Sequence analysis results revealed that the Col1 gene fragment transcripted by *K. phaffii* X33 cell and nucleotide sequence has 99% similarity with the corresponding fragment of *Bos taurus* collagen type I alpha 1 chain (COL1A1) (Fig. 4) Furthermore, it was confirmed that all of the recombinant *K. phaffii* X33 transformants which were included the expression or co-expression vectors successfully transcribed each of P4Hα, P4Hβ and Col1 genes.

According to SDS-PAGE results, it was observed that recombinant *K. phaffii* strains containing P4Hα and P4Hβ encoding genes produced protein bands of approximately 50 kDa as expected, whereas the strains, transformed with Col1 gene fragment produced the protein band of approximately a 13 kDa. Both of the 13 KDa and 50 kDa protein bands were visualized in the SDS-PAGE gel containing protein products of the transformants co-expressing the P4Hα, P4Hβ, and Col1 genes. This results proven that the COL1A1 gene fragment, and α and β subunits of the P4H enzyme were successfully expressed in the same *K. phaffii* strains.

In this study, the concentrations of proteins expressed and co-expressed by all recombinant *K. phaffii* strains were determined by the His-Tag ELISA method. The concentrations of rP4Hα and rP4Hβ and rCol1 proteins, expressed individually by recombinant strains were 10,69 µg/L, 10,74 µg/L, 8,61 µg/L, respectively, while the concentrations of co-expressed rP4Hα/P4Hβand rP4Hα/P4Hβ/rCol1 proteins were 7,82 µg/L and 5,02 µg/L, respectively.

There are several studies in the literature aiming for the co-expression of collagen gene fragments and subunits of PH4 enzyme from different animals in the recombinant *K. phaffii* strains. For example, chicken type II collagen gene and chicken prolyl-4-hydroxylase α and β subunits were simultaneously co-expressed in *Pichia pastoris* (*K. phaffii*) GS115 host cells [30,10]. Produced recombinant non-hydroxylated gelatin derived from mouse type I and rat type III collagen gene fragments in methylotrophic yeast *K. phaffii* host cells. co-transformed the α and β units of the human P4H enzyme, and the human collagen α1 (III) (COL3A1) gene into *K. phaffii* to perform post-translational hydroxylation of proline residues in COL3A1 polypeptide. In another study, human collagen A1 (III) fragment was co-expressed with viral prolyl 4-hydroxylase A085R in *E. coli* to use in biomedical and biomaterial applications were co-expressed the P4H tetramer and nonfibrillar procollagen polypeptide derived from the *Chondrosia reniformis* sponge in the *K. phaffii* host cells and determined the percentage of hydroxylated prolines in the recombinant procollagen as 36.3% by mass spectrometry.

In this study, unlike previous studies, the alpha and beta subunits encoding prolyl 4-hydroxylase and a 400 bp fragment of the bovine COL1A1 gene were inserted into the vector pPICZαA using multimer ligation method and the co-expression of the target genes was successfully carried out in *K. phaffii* X33 strain.

5. CONCLUSION

As a result, the difficulties in the production of natural collagen extracted from animal sources to standard quality and with different mechanical properties limit its use in many areas, especially in biomedical applications. This has stimulated the production of non-animal collagen-like products with desirable biomechanical properties by modifying a collagen sequence. Recombinant DNA technology enables the production of relatively modified collagen that mimics animal-derived collagen. *K. phaffii* is one host organism with excellent properties, especially for recombinant protein production in industrial applications. In this study, a 400 bp collagen fragment on the bovine COL1A1 gene and subunits of the P4H enzyme-coding genes were integrated into the pPICZαA vector using the in vitro multimer ligation method and this co-expression vector was transformed into the *K. phaffii* X33 strain so that all target proteins were expressed simultaneously in the same host cell. Although the expression capacities of recombinant strains were found to be relatively low, this problem can be significantly overcome by optimizing the protein production conditions in further studies.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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