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Cloning, Expression and Purification of Creatininase From *Pseudomonas Pseudoalkaligene KF707* in *E. coli.*

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ABSTRACT

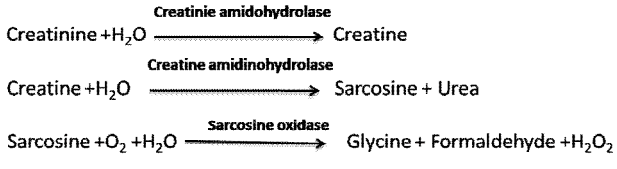
Creatinine amidohydrolase (EC 3.5.2.10) catalyzes the reversible conversion of creatinine to creatine. Creatininase in combination with other enzymes is used for detection of creatinine in serum and urine which is of significant value for detection of renal, muscular and thyroid functions. The aim of this study was to produce recombinant creatininase enzyme in *E. coli* expression system to use it in creatinine assay kit. The *pseudomonas pseudoalkaligene KF707* creatininase gene has been optimized and synthesized already. Subsequently, it has been subcloned into the pET28 expression vector then the expression vector has been transformed into the *BL21 (DE3)* cell and induced by IPTG, afterwards the expression has been evaluated using SDS-PAGE and western blot. The recombinant protein has been purified by Ni-NTA agarose resins and enzyme activity has been analyzed. A sharp 29kDa protein band has been observed on SDS-PAGE and confirmed by western blot. More than 40% of *E. coli* total protein was recombinant creatinase, The recombinant enzyme was purified with approximately 100% yield. The enzyme activity analysis showed that recombinant enzyme has 14 unit/ml activity. Recombinant *p. pseudoalkaligene KF707* creatininase was produced for the first time and its good production yield confirmed that *E. coli* was an efficient expression system for its production.

Keywords: Pseudomonas pseudoalkaligene KF707, Creatininase, Creatinine assay, Recombinant protein

INTRODUCTION

Dialysis and kidney transplantation are currently available and are considered as the essential treatments for patients with kidney-related diseases in order to prolong their lives [1]. The creatinine concentration in plasma is proportional to individual muscle mass. creatinine is filtered out of blood by the glomeruli in the kidney then excreted into urine without tubular reabsorption; so the creatinine clearance estimated from blood and urinary creatinine levels are important for renal function evaluation [2]. The creatinine concentration in serum may rise to extremely high levels above the normal ranges of serum creatinine and urine creatinine which typically fall in the range of 35-140 µM and 71-265 µmol day^{-1} kg^{-1}, respectively [3]. Therefore detection of creatinine in serum and urine is of significant value for diagnostic of renal, muscular and thyroid functions [4]. In mammals, creatinine derived non-enzymatically from creatine and creatine phosphate (a source of high energy phosphate group in muscle) at a constant rate [5].

Although there is chemical method for clinical diagnostic analysis of creatinine, this method has the disadvantage of poor specificity for creatinine. Not only accuracy, but also the precision of creatinine measurements may significantly improve when enzymatic methods are employed [4,6]. Today,among all the routine assays, enzymatic method which uses the reaction sequence shown below, is the most specific one:



The hydrogen peroxide generated in the above reaction sequence can be measured spectrophotometrically. Other enzymatic systems using creatinine deaminase [7], which

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converts creatinine to N-methylhydantoin and ammonia (with various options for measuring ammonia), and creatininase [8], (with NADH measured at 340 nm after a creatine kinase reaction sequence), have found little acceptance in clinical laboratories[9]. Creatinine amidohydrolase (EC 3.5.2.10) catalyzes the reversible conversion of creatinine to creatine and is the first step of creatinine metabolizing pathway found in some bacteria. This pathway plays a critical role in bacterial degradation of creatinine. In bacteria creatinine is a source of carbon and nitrogen. This enzyme in combination with creatine amidinohydrolase (EC 3.5.3.3) [10] and sarcosine oxidase (EC 1.5.3.1) [11] is used for enzymatic measurement of creatinine [12,5].

In recent years, genome sequence of some organisms has been determined and their coding sequence, rRNA, and tRNA have been predicted. The complete genome sequence of *pseudomonas pseudoalcaligenes KF707* has been already reported. This genome analysis has been shown to have 6512 coding sequences, 81 tRNA, and 27 rRNA [13], one of these coding sequences was recognized as creatininase which was 96% identical to *pseudomonas putida* Creatininase. These genes are proper candidates for recombinant production of some proteins and enzymes.

In this study, in order to produce creatininase with the purpose of assaying creatinine, synthesized optimized gene of *pseudomonas pseudoalkaligene KF707*creatininase was cloned in pET28a then transformed to *BL21 (DE3)* and its expression and purification was investigated. The results showed that recombinant creatininase was expressed efficiently (40% of total protein) and 60% of this enzyme was in soluble form.

OBJECTIVES

In this study, the production of recombinant creatininase from *pseudomonas pseudoalkaligene KF707*was conducted for the first time and the analysis of its expression, purification and activity were performed.

MATERIAL AND METHODS

Materials

The *E. coliDH5a* was used as the host-vector system. The *E. coli BL21 (DE3)* (novagen) was used as the hosts for protein expression. pET-28a (+) plasmid (Novagen, CA) was used to construct the expression vector. Kanamycin Sulfate was from duchefa co. (Haarlem, The Netherlands) and Ni-NTA purification resine purchased from Qiagen. pierce ^{TM} unstained protein MW marker (thermofisher scientific, catalogue number: 26610).The restriction enzymes NdeI and XhoI and DNA ligation kit, DNA marker, 2X Taq PCR master mix, and protein molecular weight marker were obtained from Thermo Fisher Scientific. The gene was synthesized by Generay Biotech Co. (Shanghai)and the primers was synthesized by bioneer Corporation (korea), Anti poly-histidine antibody-HRP was purchased from Sigma-Aldrich Chemie GmbH (Germany).

BIOINFORMATICS STUDY

The *pseudomonas putida* creatininase (uniprot id: p83772) blast was done by protein blast program from ncbi and multiple alignment of highly similar creatininases (CRNs) was conducted followed by phylogenetic tree of creatininase protein. Based on these data, the *pseudomonas pseudoalkaligenes KF707* creatininase which has the most similarity with *pseudomonas putida* CRN was selected because it performs proper function and is used in creatinine assay kit, The complete nucleotide sequence of *KF707* creatininase gene was retrieved from genebank (accession number: AJMR01000186.1) and the genetic codons of wild type creatininase gene (uniprot id: L8MLM8) from these genome sequence, encoding 260 aa were optimized based on *E. coli* codon usage table. The optimized gene was synthesized and cloned into pGH cloning vector between NdeI and XhoI restriction enzymes by Generay Biotech Co. (Shanghai).

Construction of Recombinant pET28-CRN Plasmid

The pET28 plasmid DNA was prepared using miniprep kit digested by NdeI and XhoI restriction enzyme. Moreover, the gene was digested by these enzymes and gel extracted gene was ligated into pET28a vector which fuse an N-terminal his tag to the target gene. The recombinant plasmid pET28-creatininase was transformed to *E. coli DH5α* strain by chemical transformation and the gene cloning was confirmed by colony PCR using T7 promoter

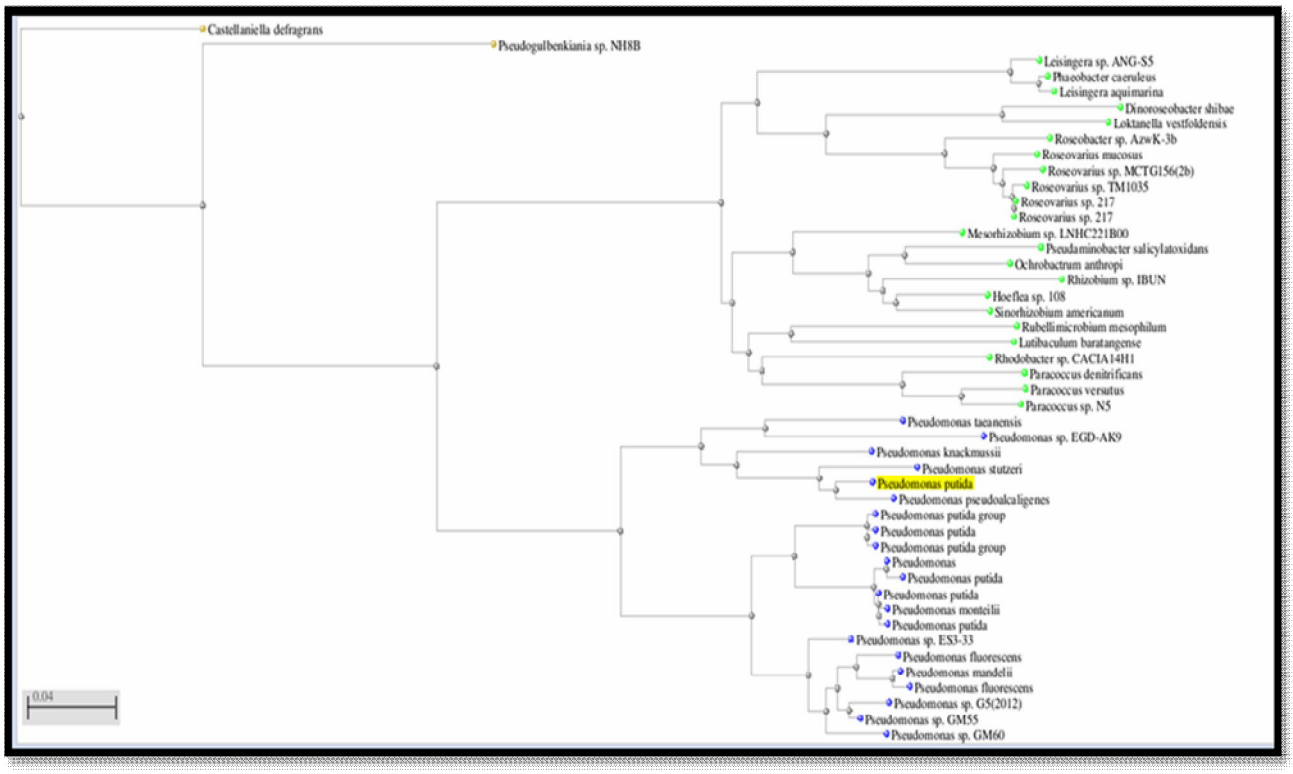

Fig. 1. Phylogenetic tree of highly similar creatininase genes. This tree obtained from *p. putida*creatininase (p83772) blast.

and T7 terminator primers, double digestion and nucleotide sequencing.

To perfom the protein expression analysis, the recombinant pET28-CRN plasmid was transformed to *E. coli BL21 (DE3)* strain by chemical transformation, the empty plasmid pET28a plasmid was used as control.

Protein Expression and its Analysis by SDS-PAGE Electrophoresis

E. coli BL21 (DE3) strain contain T7 RNA polymerase gene under the control of lacuv5 promoter. A single colony of transformed BL21contaning pET28-crn was incubated overnight on shaking incubator in 5 ml LB broth medium containing kanamycin (50 µg ml^{-1}) at 37 °C with constant shaking (150 rpm). The next day 10 ml of fresh LB broth containing kanamycin inoculated with 150 µl of overnight culture. The culture was grown at 37 °C with vigorous shaking until the OD_{600} of the culture reached 1. Then IPTG (isopropyl-β-D-thiogalactopyranoside) from Thermo Fischer Scientific was added to a final concentration of 1 mM to induce protein expression then the incubation at 37 °C with shaking (150 rpm) was continued for 4 h or overnight to analyze the effect of induction time in expression efficiency. The cells were harvested and disrupted by grinding using liquid nitrogen. The cell lysates were analyzed for total (soluble and insoluble) and soluble fractions by 12% SDS-PAGE electrophoresis.

WESTERN BLOT ANALYSIS

Total and soluble fraction of bacterial lysate resolved by 12% SDS-PAGE and then transformed to a nitrocellulose membrane using Biorad system. Membrane was then blocked by 5% skim milk in TBS-T then immunoblotted with anti his-tag HRP conjugated antibody at a 1:2000 dilution. Protein bands were developed using DAB (3,3'-diaminobenzidine) and H_2O_2 in PBS containing $NiCl_2$ as enhancer.

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Kf707           ATGAGCAAGAGCGTTTTCGTGGGAGAGCTGACCTGGAAGGAGTACGAGGCCCGTGTCGCC
Optimized       ATGTCTAAGTCTGTGTTCGTTGGCGAGCTGACTTGGAAAGAATACGAAGCTCGTGTTGCG
                ***:  ***:  ** ***** **.******** *****.**.*****.** ***** **
Kf707           TCCGGCGACGCTGTGCTGATGCTGCCGGTCGGCGCCCTGGAGCAGCACGGCCACCACATG
Optimized       TCCGGCGATGCGGTGCTGATGCTGCCGGTGGGTGCACTGGAACAGCACGGTCATCACATG
                ******** ** ********************* ** **.*****.******** ** ******
Kf707           TGCATGAACGTGGATGTCCTCTTGCCCACGGCGGTCTGCCAGCGGGTCGCCGAGCGCATC
Optimized       TGTATGAACGTTGACGTGCTGCTGCCGACTGCGGTTTGCCAGCGTGTGGCGGAGCGTATC
                ** ******** ** ** **  **** ** ***** ******** ** ** ***** ***
Kf707           GGCGCGCTGGTGCTGCCGGGGCTGCAGTACGGCTACAAGTCCCAGCAGAAGTCCGGCGGC
Optimized       GGTGCGCTGGTGCTGCCGGGCCTGCAGTACGGTTACAAATCTCAGCAGAAATCTGGTGGT
                ** ****************** ********** *****.** ********.** ** **
Kf707           GGCAACCACTTCCCCGGAACCACGAGCCTGGACGGCGCCACCCTCACCGGTACGGTGCAG
Optimized       GGTAACCACTTCCCGGGTACTACTAGCCTGGACGGTGCGACCCTGACCGGCACTGTGCAG
                ** ********** **:** ** ********** ** ***** ***** ** ******
Kf707           GACATCATCCGCGAGCTGGCGCGCCATGGCGTGCGTCGGCTGGTGCTGATGAACGGCCAC
Optimized       GACATCATCCGCGAACTGGCGCGTCACGGCGTTCGTCGTCTGGTTCTGATGAACGGTCAC
                ***************.******** ** ***** ***** ***** ********** ***
Kf707           TACGAGAACTCCATGTTCATCGTCGAGGGTATCGACCTGGCGCTGCGGGAGCTGCGCTAC
Optimized       TACGAAAACTCTATGTTCATCGTTGAAGGTATCGACCTGGCTCTGCGTGAGCTGCGTTAC
                *****.***** ********** **.*************** ***** ******** ***
Kf707           GCCGGCATCCACGACTTCAAGGTGGTGGTGCTGTCCTACTGGGACTTCGTCAAGGACCCG
Optimized       GCGGGTATCCACGACTTCAAAGTTGTTGTGCTGTCTTACTGGGACTTCGTTAAAGACCCG
                ** ** ***************.** ** ******** ************** **.******
Kf707           GCGGTGATCCAGCAGCTCTATCCGGAAGGTTTCCTCGGTTGGGACATCGAGCATGGCGGC
Optimized       GCTGTGATCCAGCAGCTGTACCCGGAAGGCTTCCTGGGTTGGGACATCGAGCACGGCGGT
                ** ************** ** ******** ***** ****************** *****
Kf707           GTCTTCGAGACCTCCCTGATGCTCGCCCTCTACCCGGACCTGGTGGACCTGGACCGCGTC
Optimized       GTGTTTGAAACCTCTCTGATGCTGGCGCTGTATCCGGACCTGGTTGACCTGGACCGCGTT
                ** ** **.***** ******** ** ** ** *********** **************
Kf707           GTCGATCACCCGCCCGCGACCTTCCCGCCCTATGACGTGTTCCCGGTGGACCCGGCGCGC
Optimized       GTTGACCATCCGCCGGCAACCTTCCCGCCGTACGACGTTTTCCCGGTGGATCCGGCACGC
                ** ** ** ***** **.*********** ** ***** *********** *****.***
Kf707           ACGCCGGCACCGGGGACCCTGTCGTCGGCGAAGACGGCCAGCCGGGAGAAGGGCGAGCTG
Optimized       ACCCCGGCTCCGGGCACTCTGTCTTCTGCGAAAACCGCGTCTCGTGAAAAAGGTGAACTG
                ** *****:***** ** ***** ** *****.** ** :  ** **.**.** **.***
Kf707           ATCCTCGAGGTCTGCGTCCAGGGCATCGCCGAGGCCATCGGCACGGAGTTTCCGTCGTCC
Optimized       ATTCTGGAAGTTTGCGTTCAGGGTATCGCGGAAGCGATCGGTACTGAATTCCCGTCTTCT
                ** ** **.** ***** ***** ***** **.** ***** ** **.** ***** **
Kf707           TGA
Optimized       TAA
                *.*
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Fig. 2. Allignment of wildtype and codon optimized *KF707* creatininase.

PROTEIN PURIFICATION

The expressed protein was purified using a column of Ni-NTA agarose resin. Cell pellets harvested from 100 ml LB broth were resuspended in 15 ml lysis buffer. Then the cell pellets were sonicated for 10 min and centrifuged at 12000 rpm for 30 min. Furthermore, the supernatant was applied to Ni-NTA column. After washing with wash buffer containing 20 mM imidazole the protein was eluted by elution buffer containing 250 mM imidazole. The purity of fractions was analyzed by 12% SDS-PAGE electrophoresis.

ASSAY METHOD

Creatinine formed from creatine by the enzyme reaction was measured using the Jaffe reaction [10]. To determine

the activity of the creatininase, 0.9 ml of 0.1 M creatine in 0.05 M potassium phosphate buffer (PB, pH 7.5) was preincubated for 5 min at 37 °C and then 0.1 ml of BL_{21} bacterial lysate containing recombinant creatininase was added to the same buffer. After 10 min of incubation at 37 °C, 0.1 ml of reaction mixture was transferred to 0.9 ml of ice-cold water and then added 1.0 ml of 1 M sodium hydroxide solution & 1.0 ml of 10 g l^{-1} picric acid solution. The mixture was incubated for 20 min at 25 °C and absorbance of the resulting solution was measured at 520 nm *vs*. the blank [14]. BL_{21} bacterial lysate containing pET28 lacking creatininase gene was used in the blank.

RESULTS

Bioinformatics studies using blast showed that *pseudomonas pseudoalkaligene KF707* bacteria, whose genome was sequenced completely, has a creatininase gene which has high similarity with *pseudomonas putida* (uniprot id: p83772) with well characterized structure and function (Fig. 1). The *KF707* creatininase gene was codon optimized (Fig. 2) based on *E. coli* genetic codons and was synthesized by GENERAY Company.

Creatininase gene was subcloned into pET28a *E. coli* expression vector. Digestion of pGH cloning vector containing CRN synthesized gene with NdeI and XhoI resulted in two bands of 700 bpCRN gene and 3700 bppGH vector; besides, digestion of pET28a vector with the same enzymes led to a linear pET28a band of 5370 bp. CRN gene and linear pET28a vector were gel extracted and ligated to creation of recombinant pET28-CRN vector.

Recombinant pET28-CRN was transformed to *BL21 (DE3)* and then cloning was confirmed by colony PCR, double digestion and sequencing. To analyze protein expression and solubility of recombinant enzymes, SDS-PAGE and western blot analysis were carried out (Fig. 3). The coomassie- stained gels were scanned and analyzed using AlphaEase FC software (Alpha Innotech, USA). The results showed that creatininase was expressed in high yield (40% of the total protein) and 60% of total protein were in soluble form (Fig. 4), and the solubility percent was calculated by dividing the percent of soluble protein calculated using AlphaEase into percent of total protein calculated using AlphaEase. The AlphaEase analysis of the

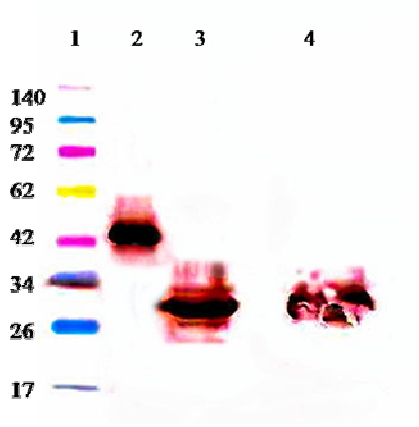

Fig. 3. Western blot analysis of expressed protein. Lane 1: prestained protein marker. Lane 2: positive control (a 45 kDa protein containing his-tag). Lane 3: crude extract containing soluble part. Lane 4: crude extract containing total protein.

effect of induction time (4 h or overnight) showed that the protein was expressed in greater amount overnight than in 4 hours. The protein was purified using Ni-NTA column with approximately 100% purity (Fig. 5). Enzyme activity was checked according to the mentioned method and the activity of the enzyme was 14 unit ml^{-1}.

DISCUSSIONS

Since the creatinine concentration is relative to muscle mass and the creatinine is glomerularly filtrated, the analysis of serum and urine creatinine concentration are important for assaying renal function [14]. Creatininase has so far been reported in several bacteria and archaea species such as *pseudomonas* and *alcaligenes*. All these enzymes have similar primary structures with sequence identity in the range from 20-96%. They enable those cells to use creatinine both as carbon and nitrogen source. In contrast, mammals possess no creatininase activity. In these organisms, creatinine arising from a non-enzymatic cyclization reaction of creatine or creatine phosphate is exclusively secreted by the kidneys. As a consequence, the creatinine concentration in blood and urine gives valuable information about renal function. One of the most powerful methods to assay creatinine is coupled enzymatic reaction

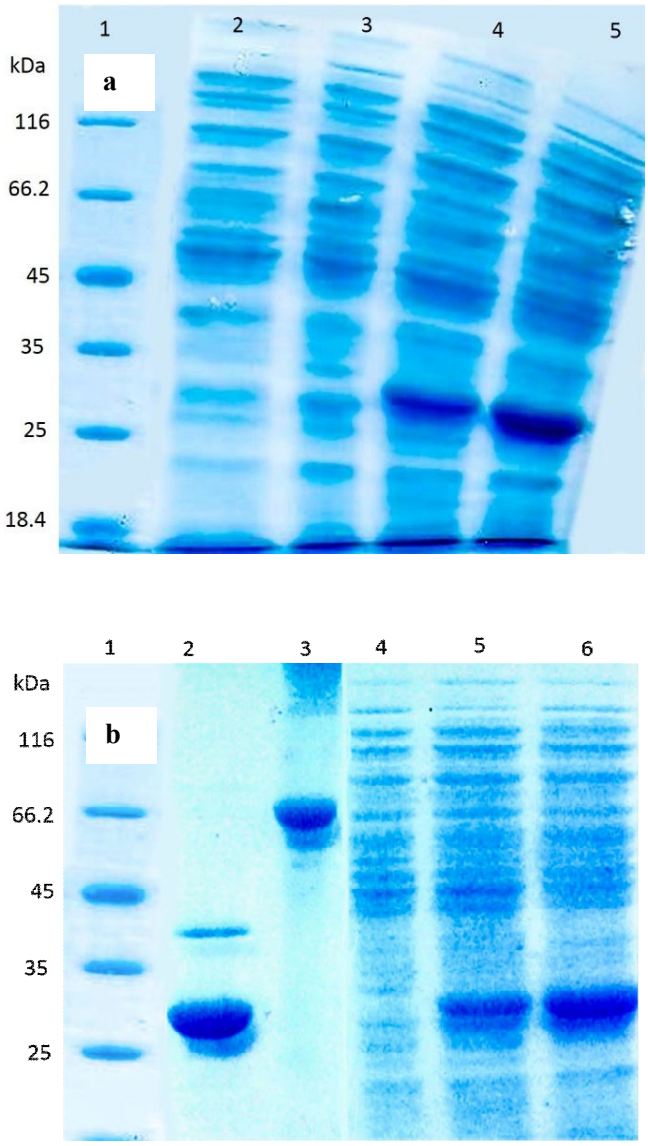

Fig. 4. SDS-PAGE analysis of expressed protein. 4a: Lane1: pierce ™ unstained protein MW marker. Lane2: crude extract of *E. coli BL21* without pET28 vector. Lane3: crude extract of *E. coli BL21* including pET28 vector without creatininase gene. Lane 4: crude extract of *E. coli BL21* induced for 4 h. Lane 5: crude extract of *E. coli BL21* induced overnight. 4b: Lane1: pierce ™ unstained protein MW marker.Lane 2: a 30 kDa protein marker. Lane 3: a 65kDa protein marker. Lane4: crude extract of *E. coli BL21* including pET28 vector without creatininase gene. Lane 5: soluble part of crude extract of *E. coli BL21*. Lane 6: total part of crude extract of *E. coli BL21*.

that is involving Creatininase, Creatinase, sarcosine oxidase and peroxidase that produce a chromogen. The color intensity of chromogen is directly proportional to the creatinine concentration and is measured photometrically. So the enzyme is highly important in medical diagnostics [15].

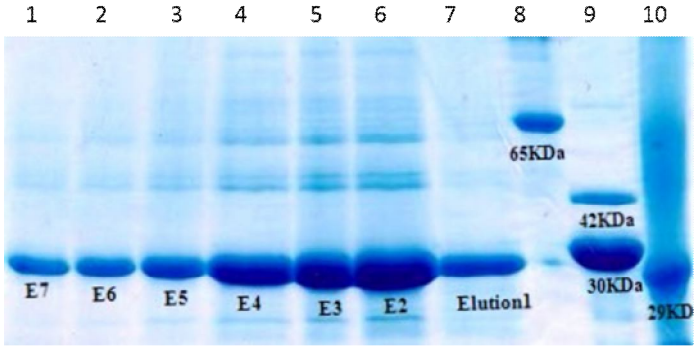

Fig. 5. Protein purification with Ni-NTA agarose resine. Lane1-7: different elution of creatininase purification. Lane 8-10: protein markers with different size.

Much attention has been directed to the production of creatininase from microorganisms [16,17,18,19,12,20] and gene coding for creatininase from *pseudomonas* [12] and *arthrobacter* [18] were cloned and sequenced. The enzymatic properties of creatininase from *pseudomonas* [20] and *alcaligenes* [16] have been already reported [3]. Creatininase was first purified from *p.putida* as a creatinine degrading enzyme [19].

The genome of many microorganisms have been sequenced completely and the function of these sequenced genome coding sequences can be proposed comparing to proteins with known structure and function On the other hand, there are a lot of translated nucleotide sequences in database in which their function has not confirmed experimentally. however, based on much identity and similarity to proteins with known function, it seems these genes can be appropriate for producing new recombinant proteins. *Pseudomonas pseudoalkaligene KF707* strain genome was sequenced completely in 2012 [13]. For find a good candidate for recombinant production, available protein and nucleotide translated sequence of creatininase in databases was gathered and aligned and their phylogenetic trees were created, so translated nucleotide sequence of *pseudomonas pseudoalkaligene KF707* creatininase (780 bp) was selected based on high similarity (96%) to *pseudomonas putida* creatininase protein sequences that its function and structure is well known.

To achieve economic viability by genetic engineering, increasing the production yield is the single most important factor in the production of biotherapeutic drugs and diagnostic kits. Many efforts have been made to construct efficient vectors which can overexpress a foreign insert DNA in specific host cells [3]. Using of *Escherichia coli* as a protein factory is a well-established alternative to purification of proteins from natural sources, on the other hand *BL21* is one of the most useful expression strains for fermentation process besides pET is one of the most widely used expression systems [21] so we can scale up creatininase production by fermentation process without changing vector or strain. Hence, the production of a recombinant creatininase in large amount is economically very essential and useful for constructing creatinine assay kit.

CONCLUSIONS

In this study, the production of recombinant *KF707* creatininase was conducted for the first time. For this reason, the optimized creatininase gene of *p. pseudoalkaligene KF707* was synthesized and subcloned into pET28a expression vector which successfully expressed the recombinant enzyme. The SDS-PAGE and western blot analysis showed its expression efficiently with high solubility. The recombinant enzyme was purified and showed a good activity. So this enzyme in combination with creatinase, sarcosine oxidase and peroxidase can be used in creatinine assay kit. The strong T7 promoter of pET28 vector allows high levels of protein expression and his-tag facilitates the purification of recombinant protein by Ni-NTA resins. Prokaryotic protein expression with pET

plasmid is one of the most efficient expression systems to produce a large amount of recombinant creatininase to develop creatinine assay kit in IRAN. Some studies have shown that enzymatic methods are preffered for evaluation of kidney function in pediatrics [6]. The analysis of expression, purification, and activity in the current study provide a simple and efficient method for producing pure active recombinant creatininase enzyme.

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