Immunology Letters 205 (2019) 78–83

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Designing a diagnostic kit for Oxalyl CoA Decarboxylase enzyme by ELISA method

Fatemeh Abarghooi -Kahaki[a], Abbas Basiri[b,*], Mojgan Bandehpour[c,d], Bahram Kazemi[a,c,d,*]

[a] Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[c] Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Departments of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
Oxalyl CoA decarboxylase
Oxalic acid
Oxalobacter formigenes
Escherichia coli
Anti body

ABSTRACT

Urinary stones are the third most commonly reported urinary tract disease. Kidney stones are one of the most common types of urinary stones that most of them (70–80%) are calcium oxalate. Oxalic acid is highly oxidized organic compounds, which found in dietary and produced by the intestinal microflora. Oxalyl CoA decarboxylase is a key enzyme and plays an important role in oxalate degradation. In this study, the Oxalyl CoA decarboxylase gene which contains an –histidine tag was cloned in pET 28a (+) and expressed in *Escherichia coli* BL21 (DE3). The purified Oxalyl CoA decarboxylase protein was injected into rabbit for immunization. The antibody against Oxalyl CoA decarboxylase protein was used in ELISA assy. eventually, this ELISA system was used for patients with calcium oxalate kidney stones. ELISA analysis of serum samples of patient white calcium oxalate kidney stones showed that 88.8% of patient was lacking in antibody against Oxalyl CoA decarboxylase. This study suggests that antibodies against oxalyl-co-decoxylase proteins are useful in the detection of urinary tract stones and It can be used to measure oxalyl CoAdecoxylase enzymes in a simple method.

1. Introduction

Renal stone is a common clinical disorder that is prevalent in advanced countries 2–3 % and in developing countries 0.5–1 % is estimated [1]. Kidney stone formation is influenced by changes in lifestyle, geographical variation, race, ethnicity and Genetic and environmental factors [2,3]. Urinary stones impose many economic and financial costs on society and can lead to kidney failure and in advanced stages of kidney degradation. About 75% of the kidney stones are calcium, which includes calcium oxalate, calcium phosphate, is a mixture of oxalate and phosphate [4,5]. Calcium stones are more common in men and the third decade is the average age of the beginning of the symptoms. *Oxalobacter formigenes* is an anaerobic, gram-negative and an oxalate metabolite bacteria in the intestine [6–9]. It uses oxalate as the sole energy source [10]. Lack of this bacterium can reduce the intake of more calcium oxalate in the colon resulting in the formation of calcium oxalate stones. *Oxalobacter formigenes* metabolizes oxalate by two enzymes; formyl-CoA transferase (FRC) and oxalyl-CoA decarboxylase (OXC) [11–13]. At first, oxalate reacts with formyl-CoA under the enzyme FRC, creating an oxalyl CoA. In the second step, oxalyl CoA is placed under the enzyme OXC, which results in carbon dioxide and formyl CoA [14]. OXC is a protein with 568 amino acids and a molecular weight of 60 kDa [15]. OXC belongs to the family of lyases, specifically the carboxy-lyases [16]. The cofactor of this enzyme is thiamine diphosphate (TPP) and binds one metal ion (usually magnesium) [17]. The enzyme consists of four identical subunits each monomer consists of three α / β domains [18]. The prevention of recurrence of the stone greatly depends to type of stones. A person who once affected stones and stones self-excreted or removed by treatment, will make another stone; possibly another 50% in the next 10 years, if no other treatments and changes in the diet are made [19]. The average rate of new stone formation in patients who have already been stone is about one stone every two or three years. For checking a person kidney stones is due to the deficiency of the enzyme OXC it should be possible to measure this enzyme. Our Studies have shown that there is no way to measure the enzymes of OXC. In the present study we use ELISA system for detection of OXC enzyme in patients. For achieving this proposes, recombinant OXC was successfully expressed. This protein was purified and injected to rabbit for Immunization. Rabbit IgG was purified and used as antigen for ELISA. Finally ELISA performance was checked out

* Corresponding authors at: Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran/Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
E-mail addresses: basiri@unrc.ir (A. Basiri), kazemi@sbmu.ac.ir (B. Kazemi).

https://doi.org/10.1016/j.imlet.2018.11.008
Received 13 September 2018; Accepted 9 November 2018
Available online 15 November 2018

and Clinical studies on patient samples considered.

2. Materials and methods

2.1. Cloning of OXC gene into pET28a (+) expression vector

DNA encoding OXC sequence (GeneBank accession number M77128.1) was synthesized into pGH plasmid (Bioneer, Korea) and confirmed by sequencing. The pGH plasmid and pET28a (+) were digested with EcoRI and NdeI. After gel Extraction, ligation was carried out with T4 DNA Ligase and the ligation reaction was transformed into *Escherichia coli* Top10 competent cell. The recombinant plasmids were confirmed by colony PCR, restriction enzyme analysis, and sequencing procedures.

2.2. Expression of the recombinant OXC protein

E. coli BL21(DE3) which was transformed with pET28a-OXC, was grown in 100 ml of LB medium (with 100 µg/ml kanamycine) at 37 °C with shaking at 250 RPM to a density of OD600 = 0.7. After that, the Isopropyl-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The cells were harvested after 2,4and 6 h and treated with lysis buffer (1 (w/v) % SDS, 0.5 (w/v)% Bromophenol Blue, 10 (v/v)% Glycerol, 0.25 M Tris-HCl pH 6.8, 5 (v/v)% B- mercaptoethanol) and 20 µl was loaded on to 10% sodium dodecyl sulfate-poly acryl amide gel electrophoresis (SDS-PAGE).

2.3. SDS PAGE and Western immunoblot analysis

Electrophoresis was carried out in 1 mm thick gels, followed by coomassie Brilliant Blue (G250) staining. For the western blot analysis, the gel was blotted on PVDF membrane by applying transfer buffer (20 mM Tris, 15 mM Glycin, 20 (v/v) % Methanol, pH = 8). The membrane was blocked with 3 (w/v) % BSA. Mouse anti-His-tag antibody (Roche 0.5 µg/ml) and goat anti-mouse HRP were used as primary and secondary antibody respectively.

2.4. Purification of recombinant His-tagged OXC

For purification of expressed His-tagged OXC affinity chromatography using Ni-NTA agarose resin was applied. The cell pellet obtained by centrifugation of 50 ml of the induced culture was resuspended in 8 ml of denaturing buffer (2 M Urea, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0) followed by shaken at 4 °C for 1 h and lysed by sonication on ice (20 s pulses 30 s). The cellular debris was removed by centrifugation at 12,000 rpm for 30 min; the supernatant was loaded on the equilibrated resin. The column was washed with 10 ml of wash buffer (40 mM imidazole, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0), the bounded proteins were eluted with 2 ml of elution buffer (250 mM imidazole, 50 mM NaH2PO4, 300 mM NaCl, pH 8.0) and dialyzed in PBS (1X) for 5 h at room temperature. Fraction of eluted -proteins were analyzed by SDS–PAGE and confirmed by dot blot.

2.5. Immunization of rabbit with recombinant OXC protein

For rabbit immunization, three four-month-old female rabbit were purchased from Pasteur Institute (Tehran, Iran) and immunized by subcutaneous injection of a 1:1 (v/v) combination of 125 (µg/ml) OXC proteins and Freund's complete adjuvant. After fourteen days the first immunization was followed by boosting with the same amount of antigen together with Freund's incomplete adjuvant. After 14 days, test bleeds were taken from their ears for ELISA. Following immunization against OXC, Antibody specificity against OXC was done by gel difussion method.

2.6. IgG ELISA assay for anti-OXC antibody

ELISA assay was done by different concentration of purified antibody and OXC protein as antigen. Microplate wells were coated with purified antibody in the concentration of 1.25, 1, 0.75, 0. 5 and 0. 25 µg/ml in carbonate –bicarbonate buffer, pH 7.2 at 4 °C over night. For each concentration of antibody, concentration of 0.75, 0.5, 0.25, and 0.125 µg/ml of purified OXC protein separately was used. Microtiter plate which was coated with antibody incubated overnight at room temperature, and PBSB (PBS containing 3% (w/v) BSA) was used for blocking for 2 h. After washing three times, antigene was added to the well and incubated for 5 h at 37 °C. After that, conjugated anti-His tag antibody was used as a secondary antibody. The immunoreaction was started by addition of 100 µl of TMB (tetramethylbenzidine). After 20 min, the peroxidase reaction was stopped by addition of 50 µl of 2 N H2SO4. Absorbance was measured at 492 nm using a microtiter plate reader.

2.7. ELISA Assay for Patients with calcium oxalate kidney stones

In order to investigate the diagnostic power of oxalyl-CoA-decoxylase anti-IgG antibodies, nine serum from infected patients and ten serum from healthy subjects were obtained from laboratory of Labafinejad Hospital stone clinic (Tehran,Iran). Subsequently all serum was analyzed for present of IgG antibody against OXC.

3. Results

3.1. Cloning of recombinant OXC

Synthetic OXC gene from *Oxalobacter formigenes* was cloned in pGH vector. The pET28a vector was successfully prepared for insertion of the OXC genes. The ligation was successfully performed and DNA encoding OXC sequence cloned into pET28a (+) expression vector. Cloning was confirmed by colony PCR (Fig. 1), enzyme digestion (Fig. 2) and sequencing analysis (data not shown). Colony PCR was done with universal primers for pET28a and a PCR product of approximately 2100 bp confirmed OXC cloning in pET28a vector. 1700 bp expected bound was observed in enzyme digestion.

3.2. Expression of the recombinant OXC gene

The prepared vector encoding OXC was transformed into the host cells for recombinant protein expression. The promoter of the recombinant plasmid was induced by IPTG at final concentrations of 0.5 mM in LB medium at 37 °C. IPTG addition induces the T7 promoter resulting in transcription of OXC gene by T7 RNA polymerase. The SDS-PAGE analysis showed that gene expression is induced for all three periods of 2, 4 and 6 h after the induction (Fig. 3). The express protein was reactive to mouse-anti-HIS tag antibody as evidenced by western blot analysis (Fig. 4).

3.3. Purification of OXC protein-His6

Proteins with Histidine-tag have affinity for Ni^{2+} that can be immobilized on chromatographic media by chelating ligands. The histidine-tagged OXC protein was purified by using Ni-NTA affinity column chromatography (Fig. 5). The eluted was used for dot blot assay. A signal was observed in dot blot analysis which confirms protein purification (Fig. 6).

3.4. Gel diffusion precipitin test

After injection of OXC protein to rabbit, an agar gel diffusion method was used for determination of precipitating antibodies to OXC protein as antigen. Positive reaction was seen between antigen and

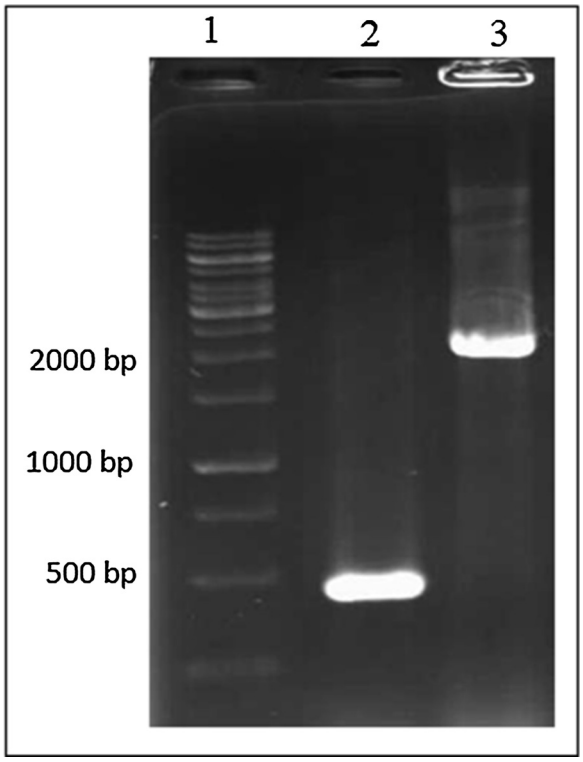

Fig. 1. Confirmation of OXC encoding gene by colony PCR: lane 1; DNA Ladder marker, lane 2 colony PCR from bacterial colony containing empty pET28a; lane 3; colony PCR of bacterial colony containing recombinant pET28a by OXC gene.

antibody (Fig. 7).

3.5. ELISA assay for Anti-OXC antibody

ELISA was performed using different concentrations of purified antibodies and different dilutions of purified OXC protein. Anyway, it was done in duplication form. The results are presented in Fig. 8.

In order to evaluate the diagnostic power of OXC-antigens against anti-IgG antibodies nine serums from affected people and also ten serums from healthy people were obtained from laboratory of Labafinejad Hospital. The presence or absence of anti-OXC in this serum was examined. At first by using ten negative serums and related formula cut off was calculated. As shown in Fig. 9, it was set at 2.9. Therefore, high values of this level were considered as positive as and lower than is negative. ELISA Numerical results of patient are shown in Fig. 10. This result of ELISA show that eight patient is negative for presence of IgG against OXC.

4. Discussion

Oxalic acid is a toxic compound that naturally present in animals, plants and fungi. High concentration of oxalate is lethal and in lower concentration it creates insoluble precipitates in combination with ions such as calcium. Human is not capable of decomposing oxalate, and it must be eliminated through the urethra or intestine. *Oxalobacter formigenes* has a key role in eliminating oxalate toxicity. Oxalate is converted into formate and CO_2 by two enzymes of OXC and FRC of *Oxalobacter formigenes.* Evidence suggests that people who don't colonize these bacteria in their intestines absorb more oxalate and prone to kidney stone formation [20,21]. So far, studies have been done to investigate the presence of *Oxalobacter formigenes* in people with kidney stones [22–24] but a study that examines the presence or absence of OXC enzymes does not exist. Investigating the presence of enzymes is very important because there may be a bacterium, but the gene associated with the enzyme is damaged and have no function. So this study first looks for a method for detecting OXC enzymes. To do this, first the OXC gene according to *E. coli* codon usage and GC content adjustment was ordered. The OXC gene which cloned in pET28a is under the control of bacteriophage T7 transcriptional signal. A high level of polymerase activity and high translation efficiency are triggered by

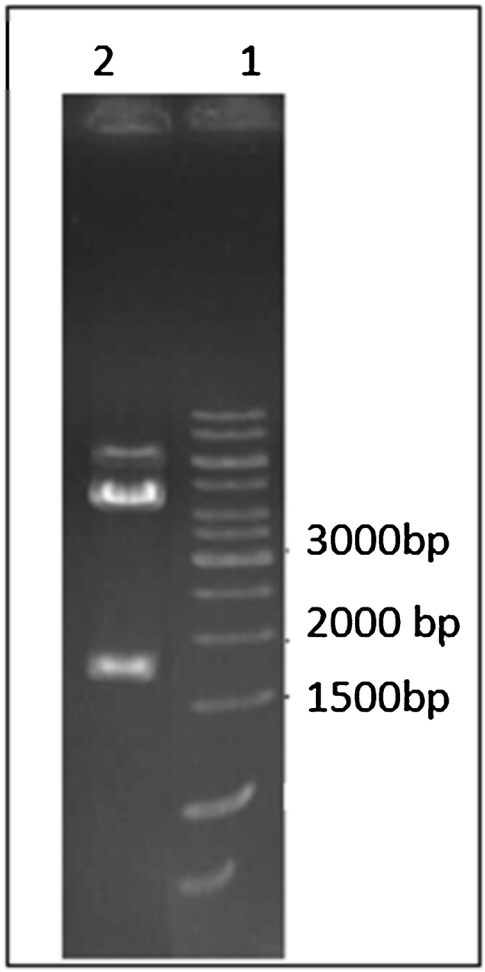

Fig. 2. Confirmation of cloning of OXC gene by restriction digestion: lane 1; DNA Ladder marker, lane 2; restriction digestion of recombinant pET28a with EcoRI and NdeI.

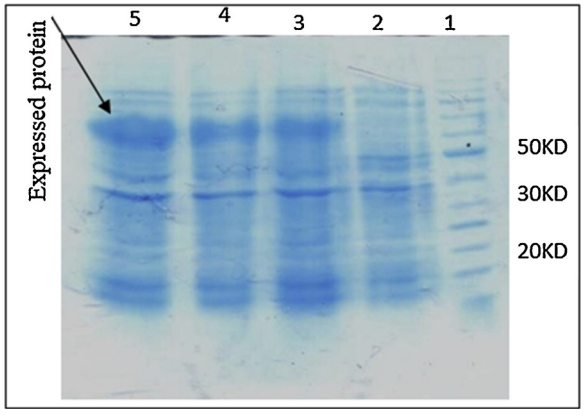

Fig. 3. A time-course analysis of OXC expression. Lane 1; protein Molecular weight marker ; lane 2 before induction; lane 3 two hour after induction; lane 4 four hour after induction; lane 5; six hour after induction.

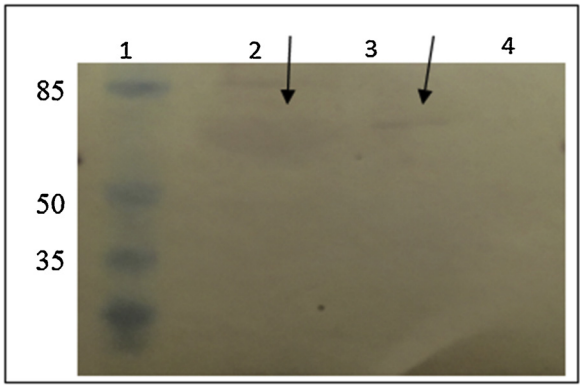

Fig. 4. Western blot analysis of the recombinant OXC expression. Lane 1; protein Molecular weight; Lane 2; expression after 6 h; lane 3; expression after 4 h; Lane 4; negative control.

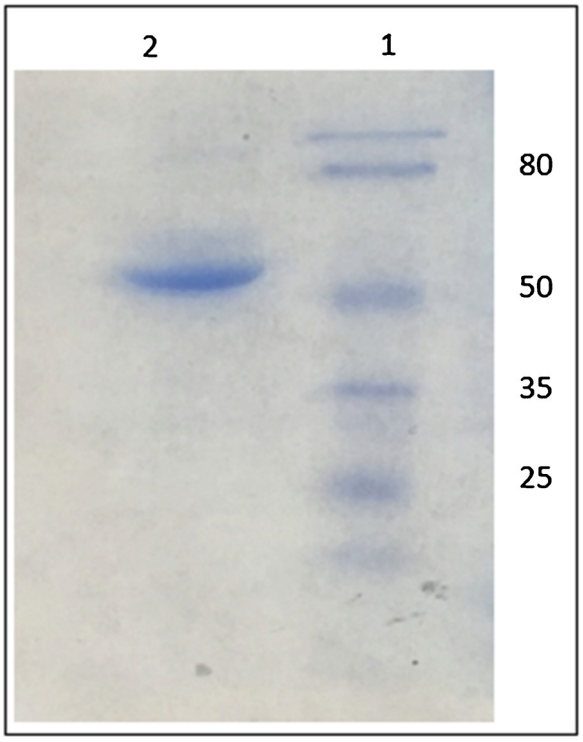

Fig. 5. Purification of OXC protein. Lane 1: protein Molecular weight; lane 2; elution of the Ni-NTA column.

translational start signals for the T7 phage gene. The expression of the protein is also induced by the chemical analogue of lactose, IPTG, which is intolerably metabolized by the host. After induction, high polymerase activity is competing with transcription by the host RNA polymerase. This issue, together with high-performance translation, creates a level of expression in which the target protein may have a major component of the cellular proteins after only a few hours. Herein OXC protein showed high level of expression So that the amount of expression in LB medium and at 37 °C and 0.5 mM concentration of IPTG was 32% of total protein. Another feature of this system is the use of histidine tag. This mark, which consists of six amino acids of histidine, is used to purify the protein by using its affinity to nickel ion. Purification of 6x recombinant OXC protein was carried out using a nickel column. Pure recombinant proteins were injected into rabbits to obtain polyclonal antibodies. Obtained antibodies were used in sandwich ELISA and ELISA was setup. Clinical studies on patient samples are being considered to examine the potential effectiveness of this ELISA in

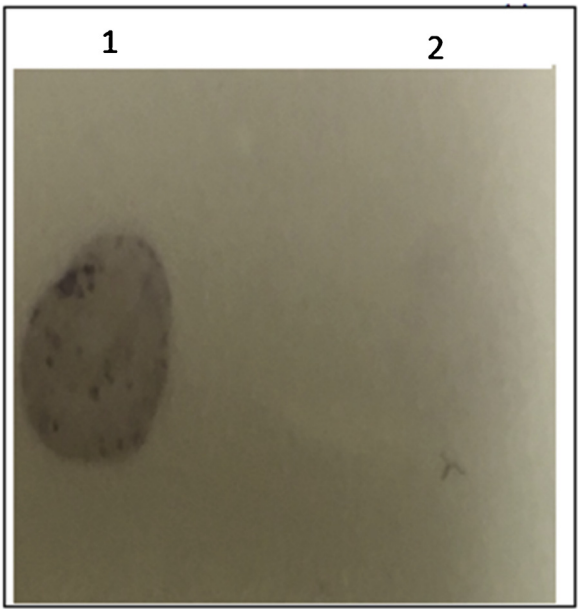

Fig. 6. Dot blot analysis of the purified OXC protein. Lane 1; eluted OXC. Lane 2; negative control.

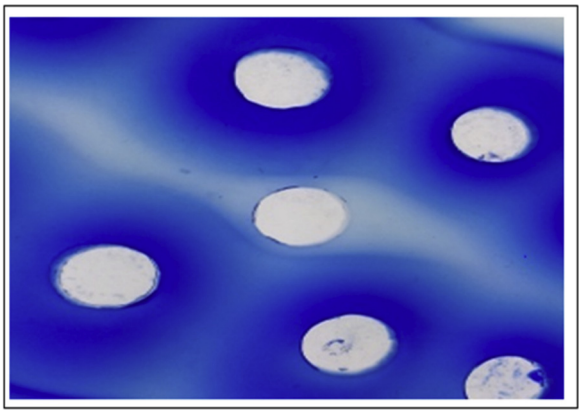

Fig. 7. Gel diffusion. Antigen(OXC) – antibody interaction.

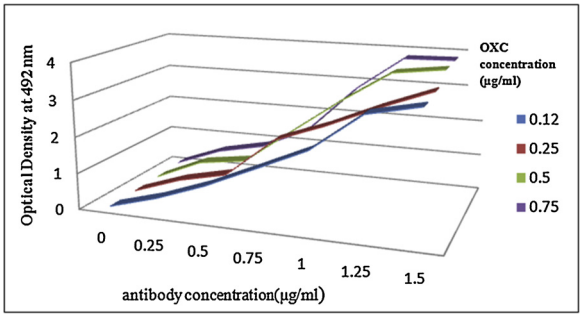

Fig. 8. ELISA at different concentration of antibody and antigen.

the diagnosis of calcium oxalate kidney stone. The positive and negative ELISA results indicate the presence and absence of OXC enzymes, respectively. The study showed that out of nine patients with calcium oxalate kidney stones, eight patients that are 88.8% had a negative response and lacked enzymes. Important factors in successful diagnosis are the simplicity, availability, results in the shortest possible time, the need for minimum facilities, and the high precision and specificity of the method. Serologic methods have been of great interest because of the use of serum and the ease of access to it, as well as the use of its

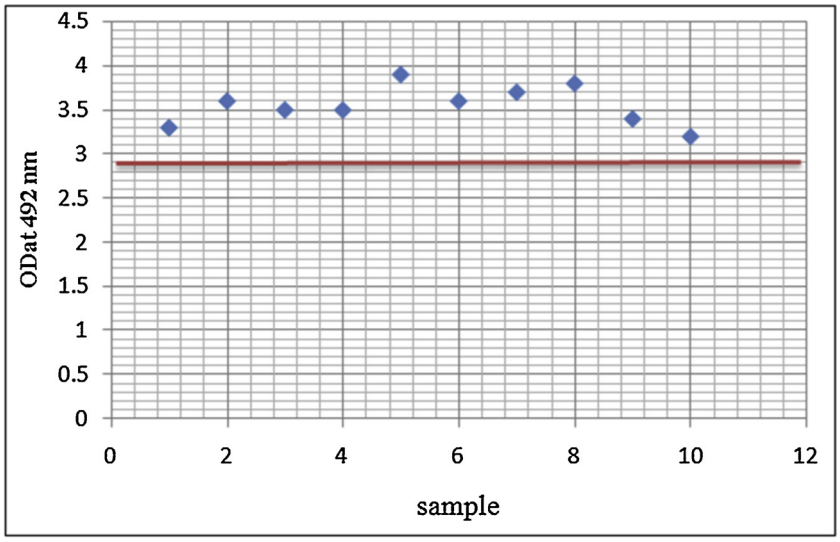

Fig. 9. Calculating cut off. cut off was set as 2.9.

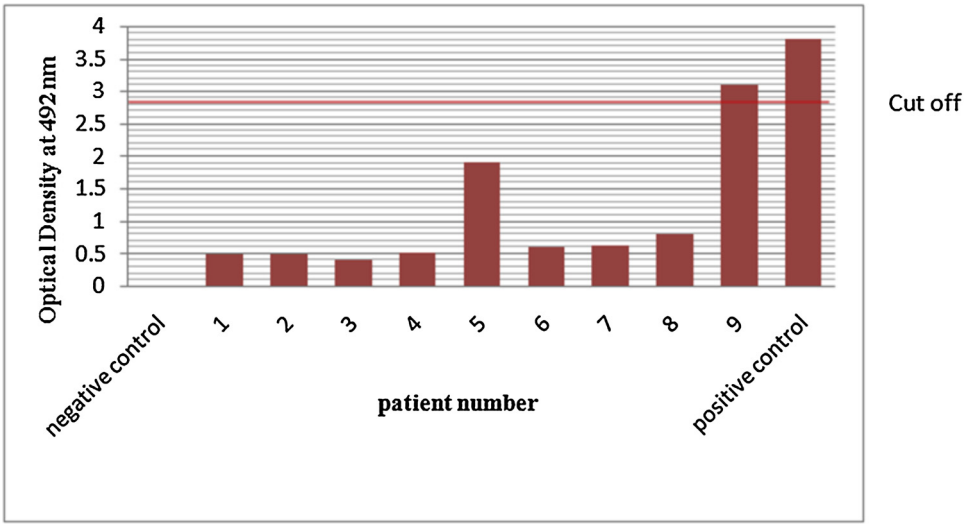

Fig. 10. ELISA Numerical results of patient with Oxalate kidney stones.

factors, such as antibodies that are very sensitive. The results of this study indicate that OXC protein have a good potential to detect calcium oxalate kidney stone. Previous studies have presented methods for measuring oxalate in biological samples. In these methods, oxalate is first separated and the quantitative measurements are carried out using colorimetric, fluorometric and chromatographic methods. These methods are often difficult, expensive, requiring a high amount of sample and skilled technician [25]. In the Boehring Mannhein method, oxalate breaks down into formate and carbon dioxide. The formate is then converted into carbonate, which NADH converts to NADH during this reaction. The amount of NADH is measured by absorbance measurement at 334 nm–340 nm or 360 nm. The problem with this method is cost, inaccuracy, reliability and insensitivity [25]. In another way, the amount of oxalate is investigated by two enzymes of OXC and FRC that convert oxalate to formate and carbon dioxide. Format produced by various methods, including colorimetrically, is measured. Since the production of formate directly correlates with the amount of sample oxalate, the amount of oxalate present in the sample can easily be measured [25]. This method requires the expression and purification of both recombinant enzymes and ultimately the amount of oxalate is measured but does not address the question of why oxalate levels are high in these patients. In our study, we initially developed a method for measuring the enzyme OXC, and eventually the amount of this enzyme was studied in patients with oxalate kidney stones. This study showed that the lack of this enzyme can play a key role in the formation of oxalate stones.

5. Conclusion

This study shows that sandwich ELISA can be an appropriate method for measuring OXC enzyme activity. OXC protein can be a promising marker for the diagnosis of kidney stone disease. The result showed that amount of this enzyme is lower in patients with oxalate kidney stones than in healthy people.

Acknowledgment

The present study was carried out in the Cellular & Molecular Biology Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran. This article was extracted from Fatemeh Abarghooi Kahaki PhD thesis and supported by Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran through Grant No 106624.

References

[1] K. Sakhaee, N.M. Maalouf, B. Sinnott, Kidney stones 2012: pathogenesis, diagnosis, and management, J. Clin. Endocrinol. Metab. 97 (2012) 1847–1860.

[2] D.J. Sas, An update on the changing epidemiology and metabolic risk factors in pediatric kidney stone disease, Clin. J. Am. Soc. Nephrol. (2011) CJN-11191210.

[3] H. Heers, B.W. Turney, Trends in urological stone disease: a 5-year update of hospital episode statistics, BJU Int. 118 (2016) 785–789.

[4] C.Y. Pak, J.R. Poindexter, B. Adams-Huet, M.S. Pearle, Predictive value of kidney stone composition in the detection of metabolic abnormalities, Am. J. Med. 115 (2003) 26–32.

[5] B.K. Auge, M.E. Maloney, B.J. Mathias, P.K. Pietrow, G.M. Preminger, Metabolic abnormalities associated with calyceal diverticular stones, BJU Int. 97 (2006) 1053–1056.

[6] C.S. Stewart, S.H. Duncan, D.R. Cave, *Oxalobacter formigenes* and its role in oxalate metabolism in the human gut, FEMS Microbiol. Lett. 230 (2004) 1–7.

[7] M.J. Allison, K.A. Dawson, W.R. Mayberry, J.G. Foss, *Oxalobacter formigenes* gen. nov., sp. nov.: oxalate-degrading anaerobes that inhabit the gastrointestinal tract, Arch. Microbiol. 141 (1985) 1–7.

[8] M.J. Allison, H.M. Cook, D.B. Milne, S. Gallagher, R.V. Clayman, Oxalate degradation by gastrointestinal bacteria from humans, J. Nutr. 116 (1986) 455–460.

[9] D.W. Kaufman, J.P. Kelly, G.C. Curhan, T.E. Anderson, S.P. Dretler, G.M. Preminger, D.R. Cave, Oxalobacter formigenes may reduce the risk of calcium oxalate kidney stones, J. Am. Soc. Nephrol. 19 (2008) 1197–1203.

[10] N. Sahin, Oxalotrophic bacteria, Res. Microbiol. 154 (2003) 399–407.

[11] A.L. Baetz, M.J. Allison, Purification and characterization of formyl-coenzyme A transferase from Oxalobacter formigenes, J. Bacteriol. 172 (1990) 3537–3540.

[12] S. Ricagno, S. Jonsson, N. Richards, Y. Lindqvist, FormylCoA transferase encloses the CoA binding site at the interface of an interlocked dimer, EMBO J. 22 (2003) 3210–3219.

[13] C.L. Berthold, P. Moussatche, N.G. Richards, Y. Lindqvist, Structural basis for activation of the thiamin diphosphate-dependent enzyme oxalyl-CoA decarboxylase by adenosine diphosphate, J. Biol. Chem. 280 (2005) 41645–41654.

[14] X. Sheng, Y. Liu, R. Zhang, A theoretical study of the catalytic mechanism of oxalyl-CoA decarboxylase, an enzyme for treating urolithiasis, RSC Adv. 4 (2014) 35777–35788.

[15] H.Y. Lung, A.L. Baetz, A.B. Peck, Molecular Cloning, DNA sequence, and gene expression of the oxalyl-coenzyme a decarboxylase gene, oxc, from the bacterium Oxalobacter formigenes, J. Bacterol. 176 (1994) 2468–2472.

[16] A.L. Baetz, M. Allison, Purification and characterization of oxalyl-coenzyme a decarboxylase from Oxalobacter formigenes, J. Bacterol. 5 (1989) 2605–2608.

[17] C.L. Berthold, P. Moussatche, N.G. Richards, Y. Lindqvist, Structural basis for activation of the thiamin diphosphate-dependent enzyme oxalyl-CoA decarboxylase by adenosine diphosphate, J. Biol. Chem. 280 (2005) 41645–41654.

[18] C.L. Berthold, C.G. Toyota, P. Moussatche, M.D. Wood, F. Leeper, N.G. Richards, Y. Lindqvist, Crystallographic snapshots of oxalyl-CoA decarboxylase give insights into catalysis by nonoxidative ThDP-dependent decarboxylases, Structure 15 (2007) 853–861.

[19] A. Trinchieri, F. Ostini, R. Nespoli, F. Rovera, E. Montanari, G. Zanetti, A prospective study of recurrence rate and risk factors for recurrence after a first renal stone, J. Urol. 162 (1999) 27–30.

[20] M.A. Azcarate-Peril, J.M. Bruno-Bárcena, H.M. Hassan, T.R. Klaenhammer, Transcriptional and functional analysis of oxalyl-coenzyme A (CoA) decarboxylase and formyl-CoA transferase genes from Lactobacillus acidophilus, Appl. Environ. Microbiol. 72 (2006) 1891–1899.

[21] S.A. Troxel, H. Sidhu, P. Kaul, R.K. Low, Intestinal Oxalobacter formigenes colonization in calcium oxalate stone formers and its relation to urinary oxalate, J. Endourol. 17 (2003) 173–176.

[22] H. Jian-zhi, Z. Xu, L. Jia-gui, Z. Yong-shang, The relationship of Oxalobacter formigenes and calcium oxalate calculi, J. Tongji Med. Univ. 15 (1995) 249–252.

[23] H. Sidhu, B. Hoppe, A. Hesse, K. Tenbrock, S. Bromme, E. Rietschel, A.B. Peck, Absence of Oxalobacter formigenes in cystic fibrosis patients: a risk factor for hyperoxaluria, Lancet 352 (1998) 1026–1029.

[24] S. Hokama, Y. Honma, C. Toma, Y. Ogawa, Oxalate degrading Enterococcus faecalis, Microbiol. Immunol. 44 (2000) 235–240.

[25] A.B. Peck, Method and kit for detection of oxalate, US Patent 5604111. 1994.