Development of Novel Point-Of-Care-Testing for Oxalate Detection in Urine Based on Oxalate-Binding Ligands.

Miss Naruerat Joybumrung

จุฬาลงกรณ์มหาวิทยาลัย
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การพัฒนาชุดตรวจ ณ จุดดูแลผู้ป่วยเพื่อวัดปริมาณออกซาเลตในปัสสาวะ โดยอาศัยลิแกนด์ที่จับจำเพาะกับออกซาเลต

น.ส.นฤรัตน์ จ้อยบำรุง

จุฬาลงกรณ์มหาวิทยาลัย
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โรคนิ่วไตเป็นโรคทางเดินปัสสาวะที่พบบ่อยเป็นอันดับสามทั่วโลกและพบมากในเขตร้อน ในประเทศไทยมีรายงานว่าพบอุบัติการการเป็นโรคนิ่วไตมากในภาคตะวันออกเฉียงเหนือถึง17% โดยโรคนิ่วไตนี้มีอัตราการกลับเป็นซ้ำประมาณ 50% ภายใน 5-10 ปี และชนิดของนิ่วในไตที่พบบ่อยที่สุดคือแคลเซียมออกซาเลต (CaOx) โดยพบว่าการขับออกของออกซาเลตในปัสสาวะที่เพิ่มขึ้นเป็นปัจจัยเสี่ยงที่สำคัญ และผู้ป่วยโรคนิ่วไตส่วนใหญ่มีภาวะออกซาเลตในปัสสาวะสูง (ออกซาเลตในปัสสาวะ >40 มก./วัน หรือ >0.46 มิลลิโมล/วัน) ดังนั้นการวัดค่าออกซาเลตในปัสสาวะจึงถูกนำมาใช้กันอย่างแพร่หลายในการประเมินความเสี่ยงของการเกิดนิ่วชนิด CaOx และใช้ทำนายการกลับเป็นซ้ำของโรคนิ่วไต ในการศึกษานี้เราเป้าหมายแรกผู้วิจัยมุ่งที่จะพัฒนาวิธีการวัดค่าออกซาเลตในปัสสาวะของมนุษย์โดยใช้สารประกอบที่ค้นพบใหม่ (Cu-nat5-BPG complex) ซึ่งมีรายงานว่าสามารถจับกับออกซาเลตอย่างจำเพาะในสภาวะ 80% ของสารละลายอะซีโตไนไตรล์และบัฟเฟอร์ HEPES 10 mM, pH 7.0. ผลการศึกษาพบว่าสารหลายชนิดที่เป็นองค์ประกอบในปัสสาวะมีผลกับการจับกันของออกซาเลตกับสารเชิงซ้อน Cu-nat5-BPG โดยถูกรบกวนอย่างมีนัยสำคัญโดยสารที่เป็นองค์ประกอบปัสสาวะทั่วไปบางชนิด เช่น ครีเอตินีน แคลเซียม และแมกนีเซียม นอกจากนี้ ระดับการขับออกซาเลตในปัสสาวะของมนุษย์มักจะน้อยกว่า 40 มก./วัน และระดับนี้ต่ำกว่าขีดจำกัดการตรวจหาของวิธีที่ซับซ้อน Cu-nat5-BPG ดังนั้นผู้วิจัยจึงเปลี่ยนเป้าหมายวิธีการการวัดค่าออกซาเลตในปัสสาวะโดยใช้เอนไซม์ออกซาเลตออกซิเดส (Oxalate oxidase, OxOx) ควบคู่ไปกับฮอสแรดิชเปอร์ออกซิเดส (Horse radish peroxidase, HRP) โดยผู้วิจัยศึกษาการโคลนและแสดงออกของ recombinant barley oxalate oxidase (rBarley-OxOx) ในยีสต์ Pichia pastoris พบว่ามีการแสดงให้เห็นว่ามีการแสดงออกของ rBarley-OxOx สำเร็จใน P. pastoris และโปรตีนลูกผสมที่มีการแสดงออกได้รับการยืนยันว่าเป็น rBarley-OxOx โดย mass spectrometry อย่างไรก็ตาม ผลผลิตของ rBarley-OxOx นั้นค่อนข้างน้อย และไม่มีความเป็นไปได้ที่จะยกระดับสำหรับการใช้งานที่ใหญ่ขึ้นในอนาคต สุดท้ายผู้วิจัยได้เปลี่ยนแผนการศึกษาอีกครั้งจากการผลิต rBarley-OxOx เป็นการซื้อ OxOx จากการสกัดและบำให้บริสุทธิ์จากต้นอ่อนข้าวบาร์เลย์ จำหน่ายโดยบริษัท Roche Diagnostics เราซื้อ OxOx และสร้างวิธีการตรวจหาออกซาเลตในตัวอย่างปัสสาวะ โดยทดสอบวัดความเข้มข้นของออกซาเลตในตัวอย่างปัสสาวะ 191 ตัวอย่างที่เก็บจากจังหวัดมหาสารคาม ประเทศไทย ผลการศึกษาพบว่าระดับออกซาเลตในปัสสาวะในกลุ่มที่ไม่มีนิ่วไต (NS-group, n=122) ต่ำกว่าในผู้ป่วยนิ่วไตอย่างมีนัยสำคัญ (กลุ่ม KS, n=69) และระดับออกซาเลตในปัสสาวะที่วัดโดยวิธี OxOx มีความสัมพันธ์เชิงบวกกับระดับออกซาเลตในปัสสาวะที่วัดโดยวิธี HPLC โดยสรุปการศึกษาในปัจจุบันแสดงให้เห็นว่าวิธีเอนไซม์ OxOx เป็นวิธีที่เจาะจงและมีประสิทธิภาพในการวัดค่าออกซาเลตในปัสสาวะมากกว่าวิธีที่ใช้สารประกอบ Cu-nat5-BPG นอกจากนี้พบว่าการโคลนและการแสดงออกของ rBarley-OxOx ของพืชในยีสต์เป็นไปได้ในห้องปฏิบัติการ แต่ไม่แนะนำให้ใช้ในเชิงพาณิชย์ต่อไปเนื่องจากผลผลิตค่อนข้างต่ำ

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Naruerat Joybumrung : Development of Novel Point-Of-Care-Testing for Oxalate Detection in Urine Based on Oxalate-Binding Ligands. . Advisor: Asst. Prof. CHANCHAI BOONLA, Ph.D. Co-advisor: Assoc. Prof. Alain Jacquet, Ph.D.

Kidney stone disease is the third most common urinary tract disease worldwide that is more prevalent in the tropics. In Thailand, the highest stone prevalence up to 17% is reported in the Northeast. The disease is highly recurrent with a recurrence rate of about 50% within 5-10 years. The most common type of kidney stones is calcium oxalate (CaOx), and an increased urinary oxalate excretion is an important risk factor. Most of kidney stone patients have a hyperoxaluria condition (urinary oxalate >40 mg/day or >0.46 mmol/day). Therefore, urinary oxalate measurement has been widely used for estimating the risk of CaOx stone formation and predicting the stone recurrence. In this study, we first aimed to develop the new method for oxalate measurement in human urine using a newly discovered compound (Cu-nat5-BPG complex) that was shown to specifically bind to oxalate in condition 80% acetonitrile aqueous solution buffered with 10 mM HEPES pH 7.0. Due to the complex matrix in urine sample, we found that the binding of oxalate to Cu-nat5-BPG complex was significantly interfered by some common urinary substances, i.e., creatinine, calcium, and magnesium. Moreover, the level of oxalate excretion in human urine is usually less than 40 mg/day, and this level was below the detection limit of the Cu-nat5-BPG complex method. We, therefore, shifted the goal to establishment of urinary oxalate measurement using oxalate oxidase (OxOx) enzyme coupled with horse radish peroxidase. We cloned and expressed the recombinant barley oxalate oxidase (rBarley-OxOx) in yeasts, *Pichia pastoris*. Our data showed that we successfully cloned and expressed the rBarley-OxOx in *P. pastoris*, and the purified recombinant proteins were confirmed to be rBarley-OxOx by mass spectrometry. However, the yield of rBarley-OxOx was relatively small, and it was not feasible to upscale for the further use. We had changed the study plan again from producing our own OxOx to buying the OxOx that commercially available. We purchased the OxOx enzyme (purified from barley seedings) from the Roche Diagnostics and established the procedure for oxalate detection in urine samples. Oxalate concentrations were measured in 191 urine samples collected from Maha Sarakham Province, Thailand. The result found that the urinary oxalate level in non-stone former (NS-group, n=122) was significantly lower than that in the kidney stone patients (KS-group, n=69). Urinary oxalate level measured by the OxOx method was positively correlated with the urinary oxalate level measured by the HPLC method. In conclusion, the present study demonstrated that the OxOx enzymatic method was a more specific and effective method for measuring urinary oxalate than the Cu-nat5-BPG complex method. Based on our findings, cloning and expressing the plant OxOx in yeasts was laboratory feasible, but it was not recommended for further commercial use mainly due to a relatively low yield.

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Chapter 1

Introduction

The title

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(English): Development of Novel Point-Of-Care-Testing for Oxalate Detection in Urine Based on Oxalate-Binding Ligands.

1. Background and Rationales

Kidney stone disease or nephrolithiasis is a condition that stones are formed in the kidneys. Statistically, kidney stone disease is the 3rd most frequent urological disease worldwide [1], and the incidence of nephrolithiasis is progressively increased in both developed and developing countries, especially in the tropics [2]. In Thailand, prevalence of stone disease is considerably high, especially in the northeastern region, where the stone prevalence is up to 17% [2, 3]. The stone disease is more prevalent in men than women. The male-to-female ratios vary across countries ranging from 3.13:1 in Germany to 1.15:1 in Iran [4]. In Thailand, the male-to-female ratio was found at 1.1 to 1.2:1 [4]. Stone disease is highly recurrent causing a difficulty in clinical management. The recurrence rate after the first kidney stones is reported around 50% within 5-10 years [5]. Several times of stone recurrence progressively damage the kidneys and increase the risk of chronic kidney disease and end-stage of renal disease [6]. In addition, kidney stone disease is associated with developments of many chronic conditions such as cardiovascular disease [7], diabetes, hypertension [8] and metabolic

syndrome [9]. Therefore, kidney stone disease is one of the public health problems that reduce the quality of life of Thai population. Development of accurate diagnostic tools and effective therapeutic regimens are need to reduce the prevalence of stone disease and increase the quality of life of the patients.

Kidney stones are heterogeneous, and they are classified by their mineral composition. The main type of kidney stones is calcium oxalate (CaOx) accounted for 60-80% of all stones, followed by uric acid (10-20%), calcium phosphate (10%), cystine (1%) and struvite stones (1-5%) [10]. Formation of stones depends on concentrations of substances (stone promoters and inhibitors) in the urine. High level of stone promoters (e.g., oxalate, calcium, uric acid, and phosphate) together with low level of stone inhibitors (e.g., citrate, potassium, and magnesium) in urine are the diving factors of lithogenic crystal formation and stone development.

Oxalate is the most important factor for CaOx crystallization. High level of urinary oxalate excretion (> 40 mg/day or > 0.46 mmol/day) [11] or hyperoxaluria is a known cause of CaOx kidney stone disease . Therefore, measurement of oxalate content in urine is widely accepted for evaluating the risk of kidney stone formation and predicting CaOx stone recurrence. Up to now, there are many studies reporting about oxalate determination in food and clinical samples (such as serum and urine). In kidney stone patients, urinary oxalate measurement is essential for preventing recurrent stone development. In general peoples or non-stone forming individuals, oxalate concentration in urine can be used to estimate the risk for CaOx stone formation. If the urinary oxalate level is high, preventive measures for reducing the risk of stone formation, such as increased fluid intake, avoiding high oxalate diets and increased consumption of citrus fruits, must be implemented. The current techniques for determining oxalate in urine samples are divided into two main groups, non-enzymatic assay and enzymatic assay.

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The non-enzymatic techniques for urinary oxalate measurement include high performance liquid chromatography (HPLC) [12], capillary electrophoresis (CE) [13], ion chromatography [14] and gas chromatography [15]. In our research group, we recently developed a new method for indirectly detecting oxalate in the urine based on precipitation of CaOx crystals, called calcium oxalate crystallization index (COCI) [16]. The most well accepted non-enzymatic technique for oxalate measurement is HPLC, but it is complicated and expensive. Also, it requires multi-step sample preparation, sophisticated equipment, and operating skill. To address these issues, we proposed to develop a novel non-enzymetic assay for oxalate detection based on oxalate specific-binding ligands. The assay was expected to be rapid, sensitive, specific, accurate, economy, and user-friendly, and it could be used as point-of-care testing (POCT).

Cu-nat5 is a novel complex of two copper ions and nat5 ligand developed by Assistant Professor Chomchai Suksai, Ph.D. at Burapa University and her team. It was designed for selectively binding to anions, and it showed selectively binding to oxalate under specific condition. The Cu-nat5 was synthesized from the ligand L and $Cu(ClO_4)_2$ [17] reported by Nattawat Chatphueak et al. at faculty of Science, Burapha University, and its structure was characterized (Fig. 1). The selectivity for oxalate binding was tested with 38 anions, based on indicator displacement assay (IDA) using bromopyrogallol red (BPG) as a colorimetric indicator (Fig. 1). It turned out that this Cu-nat5 complex is highly specific to oxalate. Cu-Nat5 was combined with BPG to produce the ensemble (Cu-nat5-BPG) with blue color. After adding oxalate, the BPG dye was replaced by oxalate, and the dye was released. The solution color was rapidly change from blue to pink (Fig. 1). The pink color intensity was correspondingly related to the concentration of added oxalate. However, this CU-nat5-BPG ensemble has never been tested in urine samples that contain many biological substances at varied concentrations. We aimed to test whether these urinary substances could interfere with the oxalate displacement.

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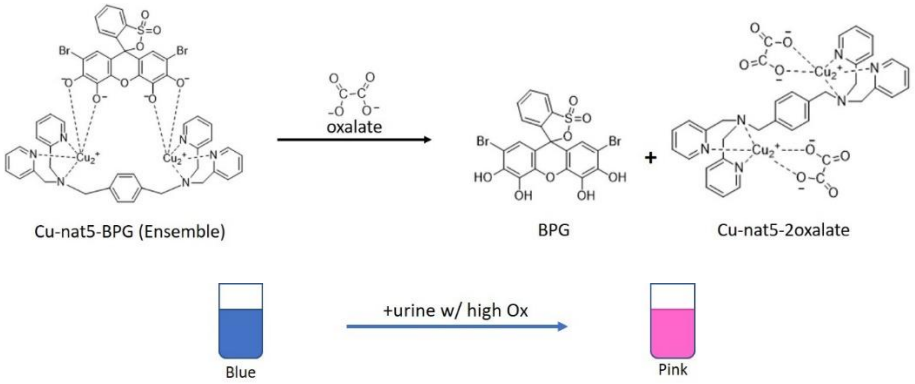

Figure 1. The principle of oxalate measurement based on indicator displacement assay. Two oxalate molecules displace the BPG dye in the ensemble (Cu-nat5-BPG). The color immediately changes from blue to pink after the displacement. Intensity of pink color is proportionated to the oxalate concentration.

In enzymatic assay, determination of oxalate content is based on two types of enzymes, oxalate oxidase (OxOx) and oxalate decarboxylase (OxD). These two enzymes are widely used in research and commercial oxalate detection kits (13). OxOx breaks down oxalate in the presence of oxygen into carbon dioxide and hydrogen peroxide, by which oxalate concentration can be measured by the amount of hydrogen peroxide produced in the reaction. OxD reacts with oxalate to produce formate and carbon dioxide. In this reaction, amount of oxalate is derived from the amount of formate. Both enzymes are widely used to detect oxalate because they are specifically reacting to oxalate and can be used in colorimetric or fluorometric quantitative measurements. In OxOx reaction, horseradish peroxidase (HRP) and its substrate are required for to quantifying amount of hydrogen peroxide. By contrast, formate dehydrogenase, nicotinamide adenine dinucleotide (NAD) and phenazine methosalfate(PMS) are required for measuring the amount of formate in OxD reaction. Generally, OxOx reaction is less complexity, easier to perform, and cheaper to develop than OxD reaction. In this study, we were interested in development of OxOx enzymatic

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assay, and we aimed to produce our own recombinant OxOx enzyme using the cloning technology.

OxOx enzyme is found in some plant species such as barley, wheat, sorghum, and banana peel. It is also found in some molds and bacteria. The plant OxOx has been extensively studied and proposed to be the best source of OxOx to be used for diagnostic application. The best characterized OxOx is the OxOx enzyme that extracted and purified from the germinating seeds of barley and wheat. In this study, we employed gene cloning tool to produce barley OxOx in yeast model, *Pichia pastoris* [18].

In this study, we aimed to develop the new testing system for oxalate measurement in urine samples. The study was divided into three parts. Part 1, we tested the interference of urinary substances in measuring oxalate concentration by IDA using the Cu-nat5 ligands and asked if this Cu-nat5-based assay was feasible for measuring oxalate in human urine samples. Part 2, we cloned and expressed recombinant barley OxOx in *P. pastoris.* Part 3, we developed our own OxOx-based system for oxalate detection in urine samples obtained from non-stone subjects and kidney stone patients. Also, urinary oxalate levels determined by OxOx and HPLC methods were compared to see whether they were well correlated or not.

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2. Keywords

Kidney stone, Nephrolithiasis, Hyperoxaluria, Oxalate detection, Oxalate-binding ligand, Indicator displacement assay, Colorimetric detection, POCT

3. Research questions

1. Could a new measurement of oxalate in urine based on indicator replacement assay (using Cu-Nat5 oxalate binding ligands) be successfully detecting oxalate in human urine?

2. Could recombinant barley OxOx enzyme be cloned and expressed in *P. pastoris*?

3. Could the procedure of OxOx based method for urinary oxalate measurement be established and if the level of urinary oxalate measured by this method be clinically useful for separating non-stone forming individuals from the CaOx stone formers?

4. Objectives

To develop a new oxalate measurement technique for detecting oxalate in urine based on non-enzymatic assay and enzymatic assay

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5. Hypothesis

We hypothesized that the new in-house oxalate measurement method could be developed to be clinically useful to separate non-stone subjects from calcium oxalate stone patients.

Chapter 2

Review related literatures

Epidemiology of kidney stone disease and clinical related

Kidney stone disease (nephrolithiasis) is a common urological disease, especially in tropical countries. During the past decades, the incidence of the disease has increased in worldwide [1]. Thailand is one of the countries in the stone belt (Figure 2.). The prevalence of the disease is high ranging from 10-17%, mainly in the northeastern Thailand [2, 3]. The major problem of kidney stones is recurrence after stone removal. It has been reported around 10–23% in the first year, 50% in 5–10 years, and 75% in 20 years [4]. The disease may also present with chronic, episodic flank pain or may even be asymptomatic [5]. There are a lot of evidence confirming that the kidney stone has a direct deleterious effect on kidney function, and it is closely associated with chronic kidney disease (CKD) and finally leads to a lethal condition, end-stage renal failure disease (ESRD). Moreover, kidney stone has been found to be associated with many other diseases, including cardiovascular disease [7], diabetes, hypertension [8] and metabolic syndrome [9].

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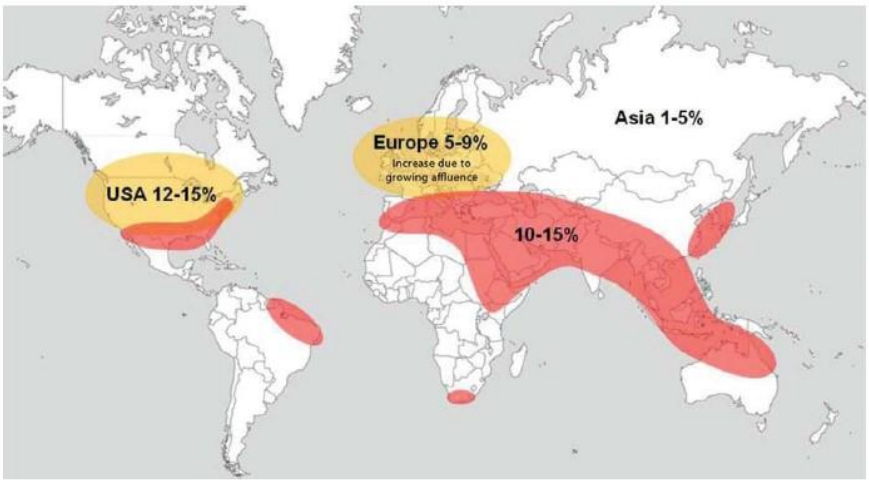

Figure 2. Stone belt (red) extends all the way around the world.

Types of kidney stones

Stone types are simply classified by major mineral content in the stones. Most of stones contain calcium, and it is usually combined with oxalate, so called calcium oxalate stone. Beside calcium stone, non-calcium stone remains a urologic problem, and it is found about 20% of all stones.

Calcium stones, the main type of kidney stones, are accounted for approximately 80% [4, 10], 50% of these are pure CaOx, about 5% are calcium phosphate (CaP), and approximately 45% are a mixture of both. Therefore, almost all of calcium stone contains oxalate as a main component. There are many factors that cause CaOx stones such as hyperoxaluria, hypercalciuria, hypocitraturia, hypomagnesiuria and low urine volume. The pH of urine that promotes formation of CaOx stones is around 5.0–6.5, whereas pH more than 5 promotes CaP stone formation.

Uric acid stone is accounted for 3-10% of all stone types. Uric acid is poorly soluble or even insoluble at urine pH lower than 5.05, hence facilitating formation of uric acid stone[4],[10]. The main etiology of uric acid stone is high consumption of purine-rich diets, especially meats, such as pork, beef, and chicken, resulting in increased excretion of urinary uric acid or hyperuricosuria. Moreover, in the condition that the ammonium is excreted lower than normal, it directly causes of acidic urine to enhance uric acid stone formation. In gout patients, both low urinary pH and uric acid stone formation are commonly found [4].

Struvite or magnesium ammonium phosphate (MAP) stones are found up to10-15% of all stones. This type of stone is formed in the alkaline urine (pH >7) [4]. The etiology of MAP stone is often caused by infection, so-called infection stone. Urinary tract infections by several urea-splitting bacteria such as *Proteus mirabilis* (most common), *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and Enterobacter. These bacteria produce urease, and the produced urease is used to degrade urea into

ammonia and CO_2. This results in a more alkalinizing urine and promote MAP crystallization and eventually stone formation.

Cystine stones is a genetic or monogenic stone. It is rare and usually found less than 2% of all stones. It is caused by genetic mutation of rBAT gene on chromosome 2 [16]. This mutation leads to abnormal reabsorption of an amino acids, including cystine in renal epithelial cell transporters, resulting in elevated level of cystine in urine. Cystine crystals are hardly dissolved in urine, especially in the acidic urine, and that leads to formation of cystine stone.

Causes of CaOx stone.

CaOx stone is the main type of kidney stones. Cause of CaOx stones can be divided into 2 main causes.

1. **Intrinsic factor** is a genetic mutation or polymorphism that favor increased excretion or calcium and oxalate leading to hypercalciuria and hyperoxaluria. In addition, other intrinsic factors such as age and sex have been reported to contribute to CaOx stone formation as well.

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Primary hypercalciuria often caused by gene mutations that are related to bone resorption, calcium absorption in the kidney and calcium metabolism. For example, Dent disease; This is a mutation in *CLCN5* gene, inherited in X-linked recessive mode. The gene encodes a kidney-specific chloride/proton antiporter. Bartter syndromes: There are 5 types, categorized by abnormal genes. This condition causes hypercalciuria and uncommon crystal deposits. Primary hyperparathyroidism; This condition cause more bone breakdown and hence elevating blood calcium that further increases calcium excretion in urine [10].

Primary hyperoxaluria (PH), it is caused by mutation in *AGXT* (PH type 1) gene or *GRHPR* (PH type 2) gene. These mutations lead to oxalate overproduction in liver and

elevated excretion though urine. In PH condition, oxalate levels in urine is very high, and it can be up to 100-200 mg/day [11]. (normal < 40 mg/day in male and female [19])

Sex and Age; CaOx stone occurs more frequently in men than in women within the age of 20–49 years in the male-to-female ratio of 2:1 to 3:1 [4]. For age, a peak of first stone episode is found between 40 and 50 years old for CaOx stone. The study in Japan found that the stone prevalence is shifted to an older age in both males and females from 1965 to 2005 [20]. Additionally, the glomerulus filtration rate is generally decreased by 5-10% after age of 35 years old resulting in reduced filtration or reabsorption in the kidneys [21], that might contribute to stone formation.

2. **Extrinsic risk factor** for kidney stone formation is mainly caused from environment and lifestyle, such as geography, climate, dietary, and water intake.

Dietary habit; Increased consumption of oxalate-rich foods lead to increased risk of kidney stone formation. Foods containing high amounts of oxalate include spinach, rhubarb, almonds, and cashews etc. Exposure to some chemicals that can be metabolically converted into oxalate in the body is also another cause of hyperoxaluria, for example, ingestion of ethylene glycol (EG) and glyoxylic acid. To date, EG-induced nephrolithiasis is a well-accepted model for experimental nephrolithiasis (Figure 3.) [22, 23]. Moreover, ingestion of high sodium diets and increased vitamin C intake also lead to increased risk of CaOx stone formation. This is because high sodium increases calcium excretion and vitamain C is metabolized to generate oxalate, that further increase urinary oxalate concentrations [23].

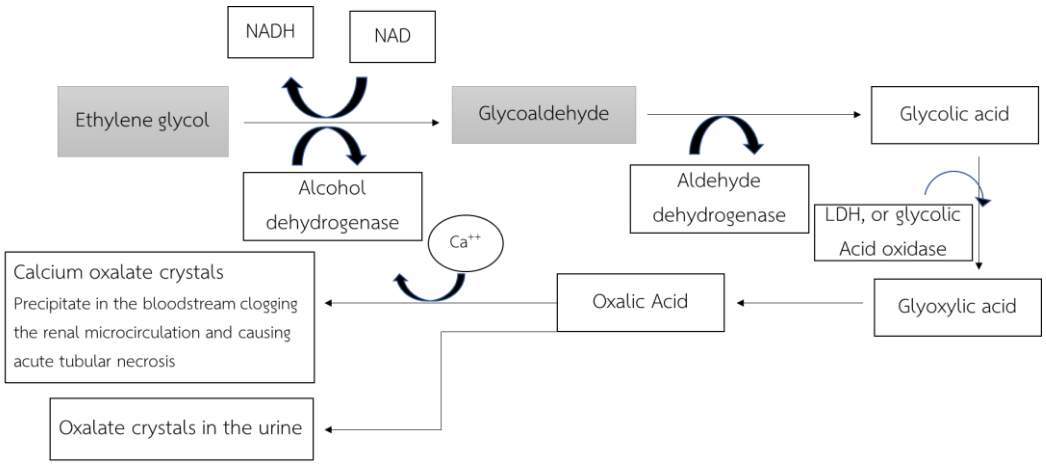

Figure 3. Pathway for metabolism of ethylene glycol to increase oxalate excretion.

The most important extrinsic cause of CaOx stone formation is insufficient intake of citrate-containing diets. Citrate is a potent inhibitor of stone formation. In renal tubule, citrate complexes with calcium to form soluble calcium citrate compound, hence reducing the concentration of free calcium in the urine and lowering the chance of CaOx crystal formation [24].

Water intake: inadequate water intake makes urine supersaturation and facilitates crystallization. The recommendation for fluid intake is at least 2 liters per day to prevent CaOx supersaturation and crystallization in urine.

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Geography and climate; Prevalence of kidney stones is quite high in the tropical region compared to the cold countries. It is simply because of the heat that causes increased fluid loss from the body (such as through sweating). It results in reduction of urine volume that further leads to urine supersaturation. Due to global warming that is indicated by a significant rise of mean annual temperature (MAT) in the U.S. [25], there are more patients come to see a doctor with kidney stone condition, even in the states that are not tropical states. It is now accepted that climate change contributes to progressively increasing of kidney stone incidence worldwide [25-28].

Mechanisms of Renal Stone Formation

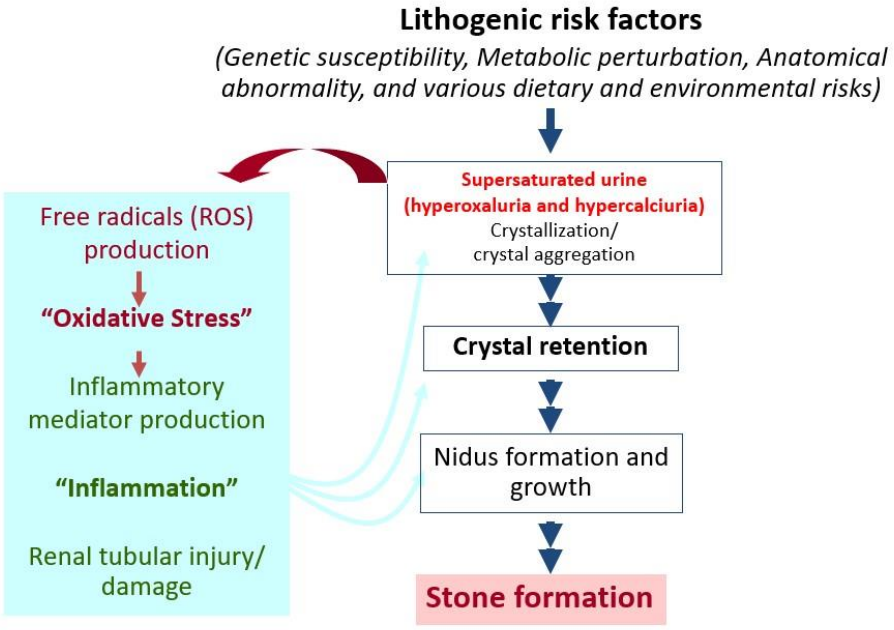

Figure 4. Key mechanistic steps in the process of kidney stone formation

Supersaturation phase is the first step and key role to drive all phase changes, which is a condition of hypercalciuria and hyperoxaluria (high concentration of calcium and oxalate in urine), often found with hypocitraturia and a low volume of urine that promote supersaturation salt between calcium and oxalate. When the concentration of calcium and oxalate ions exceeds solubility in the urine, these ions can bind to form crystals, called crystallization. The crystallization has 2 phases, consist of nucleation phase and crystal growth. The nucleation is a first phase, which is birth of new crystals and the crystal growth is a second phase, which is a growth of crystals to get a larger size. Then crystal aggregation until appropriated size and retain in the kidney. Meanwhile, excess CaOx crystals and oxalate can induce ROS production lead to oxidative stress, inflammation and renal tubular injury [29]. The tubule injury is an appropriate position for crystal attachment. Nidus (site of stone origin or crystal attachment) is then formed, grown and finally become stone [30].

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Hyperoxaluria-induced oxidative stress and kidney injury

Figure 5. Putative mechanism of oxalate-induced oxidative stress, kidney injury and crystal deposit.

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Oxalate in hyperoxaluric condition activates the renin–angiotensin–aldosterone system (RAAS). Renin upregulation and generation of angiotensin II induce aldosterone release, which in turn activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) [31]. Nox is an oxidase enzyme that is one of the most important sources of reactive oxygen species (ROS) production, especially Nox4 isoform. High oxalate and CaOx crystals increase Nox activity through p22 transmembrane subunit (Fig.5) [29, 32]. A previous study suggested that renal Nox4 activation is contributed to renal damage [33]. Oxalate also causes increased production of ROS via the mitochondria respiratory chain [34]. High level of ROS from both sources (Nox and mitochondria) activates many downstream pathways such as nuclear factor-kB (NF-kB), which is a pathway that promotes the inflammation. Normally, antioxidants are shown to effectively

inhibit ROS, but when ROS are chronically excessively produced, it leads to insufficiency of antioxidant. The excessive ROS directly injure the cells and cause tissue injury. The injured cells and newly proliferated cells have some specific features that favor crystal attachment that further leads to crystal deposit and stone formation [30].

Chemical composition of 24-hour in urine

For stone diagnosis and evaluation of risk for stone formation, 24-hour urine is a specimen of choice to be collected for measurement of levels of stone promoters and inhibitors. The normal chemical composition of urine is mainly water (approximately 95%). When the abnormal chemical composition of urine occurs, it is implying that the body has some pathogenesis. Urinary abnormalities that are mostly found in kidney stone patients are low urine volume, hyperoxaluria ,hypercalciuria and hyperuricosuria, depending on types of stones [19]. Low urinary excretion of stone inhibitors such as hypocitraturia and hypokaliuria are also frequently present in the stone patients. Table 1 shows the reference range of each lithogenic (promoter) and antilithogenic (inhibitor) substances in 24-hour urine. Urinary citrate and oxalate are key determinants for lithogenesis and antilithogenesis, respectively.

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Table 1. Chemical composition of 24-hour urine in adult non-stone formers

Measurement	Normal range in 24-hour urine (in adults)
Volume (L/day)	> 1.5 L/day
Calcium (mg/day)	<300 (M), <250 (F)
Oxalate (mg/day)	<40 (M or F)
pH	5.8 – 6.2
Phosphate (mg/day)	500–1500
Citrate (mg/day)	>450 (M), > 550 (F)
Uric acid (mg/day)	<800 (M), <750 (F)
Sodium (mmol/day)	50–150
Potassium (mmol/day)	20–100
Magnesium (mg/day)	50–150
Sulfate (mmol/day)	20–80
Ammonium (mmol/day)	15–60
Creatinine	20–24 mg/kg (M), 15–19 mg/kg (F)
Urine cystine screen	Negative

M: males, F: females

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Urinary oxalate measurement

1. Enzymatic assays

Enzymatic assays are the most commonly used methods for oxalate measurement. It is mainly because of their specificity to oxalate. Two widely used enzymes for the oxalate determination are oxalate oxidase and oxalate decarboxylase (17).

Oxalate oxidase (OxOx) (EC 1.2.3.4) is an enzyme that mostly found in plants, such as barley [35], wheat [36], banana peel [37], maize root [38] and etc. In addition, it can be found in some species of fungi and bacteria. However, the barley seeding OxOx enzyme is the best characterized one [35]. OxOx specifically splits oxalate into carbon dioxide (CO_2) and hydrogen peroxide (H_2O_2). Mostly, the enzyme activity is idirectly measured through amount of H_2O_2 production. Therefore, horseradish peroxidase (HRP) is needed to convert H_2O_2 to color product. Increased color intensity is a reflective of increased H_2O_2 production, which is proportionated to oxalate concentration. Figure 6 shows the enzymatic reactions of oxalate measurement by OxOx and HRP.

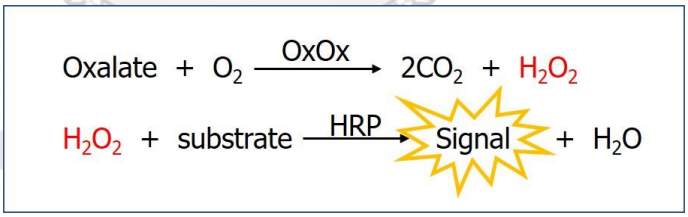

Figure 6. Principle for enzymatic assay for oxalate measurement by oxalate oxidase (OxOx) coupled with HRP

The method based on OxOx is the most frequent method used for oxalate detection. There are many studied reported the development and optimization of oxalate detection base on OxOx with various detection formats such as spectrophotometry [39], immobilized glass bound enzymes [40], and catalase model compound [41]. In

addition, some studies directly measured the amount of H_2O_2 by amperometric biosensor method [42].

Oxalate decarboxylase (OxD) (EC 4.1.1.2) is an oxalate-degrading enzyme that mostly found in fungi and bacteria [43]. The best characterized OxD enzyme is from Bacillus subtilis [43]. OxD degrades oxalate to generate formate and carbon dioxide (CO_2). Measurement the amount of oxalate from the generated formate product needs to use formate dehydrogenase as well as its cofactor and substrate, nicotinamide adenine dinucleotide (NAD) and phenazine methosalfate (PMS) (Figure 7).

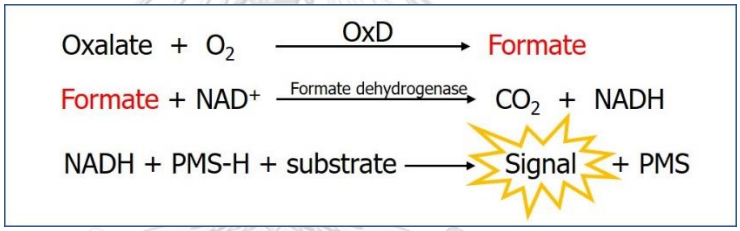

Figure 7. The principle for enzymatic detection of oxalate by oxalate decarboxylase.

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There are studies that used OxD to detect oxalate with various platforms, for instances, by spectrophotometry [44], immobilized OxD on gas-sensing electrode [45] and sensor paper-based colorimetry [46].

2. Non-enzymatic assays

High-performance liquid chromatography (HPLC); There are many studies demonstrated measurement of oxalate by HPLC technique. One of them is study by Suryavanshi et al. [12]. They used reverse phase-HPLC for oxalate analysis. The detecting wavelength for oxalate is at 237 nm, and oxalate concentration is calculated

from area under peak (Figure 8) obtained from the chromatogram (Figure 9) They found that the limit of detection of this method is of 49 ng/mL [12].

Oxalic acid content (µg/ml) = area of oxalate peak/mean of area of 6readings × 10

Figure 8. The formula for calculation of oxalate concentration from HPLC chromatogram.

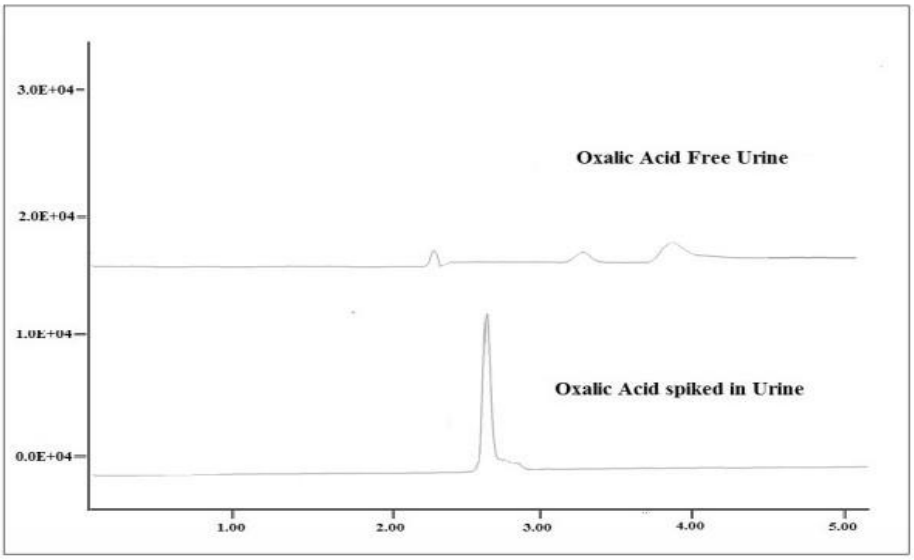

Figure 9. The HPLC chromatogram compared between non-spiked urine (oxalic acid-free urine) and oxalic acid spiked urine.

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Calcium Oxalate Crystallization Index (COCI); This COCI method is developed by our group in 2014. It does not directly measure oxalate content, but it is measured the capability of urine to crystallize CaOx by precipitation of CaOx crystals in urine samples [47]. The crystal pellets were dissolved and measured at absorbance 215 nm (Bowei Yang and co-workers, 2014). In Figure 10, it shows that COCI values are increased proportionally to the concentration of oxalate.

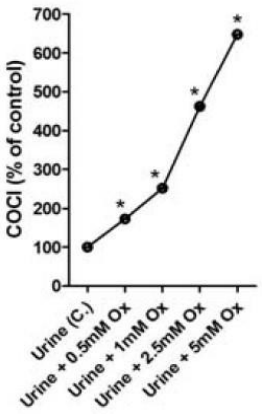

Figure 10. The effect of oxalate on COCl values

Bio-sensor; There are many studies that tries to develop chemical ligands specifically bind to oxalate in order to use for oxalate measurement. Adventage would be to reduce cost of enzyme production. Also, as the chemical binding reaction is simple, sophisticated equipment is no longer required. Up to now, there are two classical methods, direct sensing and indicator displacement assays (IDA), that have been used to design sensing system for oxalate detection (Figure 11).

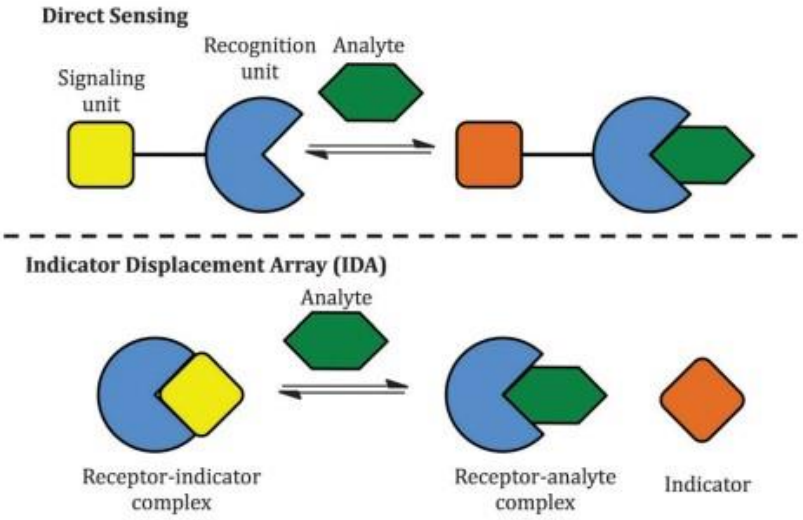

Figure 11. Two approaches to construct chemical sensors for analyte (oxalate) detection: direct sensing (top panel) and an indicator displacement assay (IDA) (bottom panel).

Direct sensing system requires a signaling unit covalently and permanently connected to a recognition unit. The analyte of interest (in our case is oxalate) binds to the recognition unit resulting in a signal change (Figure 11). The example of direct sensing for oxalate sensing is a study by Gan et al. published in 2019. They use the recognition unit that contains MnO_2 and use TMB as a colorimetric indicator (signaling unit) (Figure 12) [48].

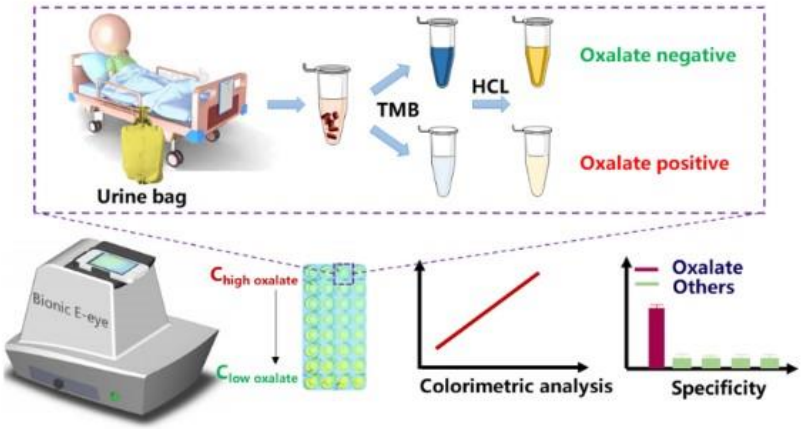

Figure 12. Schematic of TMB-MnO_2 nanosheets for colorimetric oxalate detection by bionic E-eye system.

Indicator displacement assays (IDA) is different from direct sensing, as the recognition and signaling units are separated molecules. IDA relies upon competition between the indicator and the specific substance in the receptor or ligand (Figure 10). The example of IDA for oxalate sensing is a study by Tavallali and co-workers in 2018 [49]. Cu-RB4 is a receptor-indicator complex, which shows a light blue color. Then oxalate reacts with the complex and turns the color from light blue to dark blue. The peak of maximum absorption is at 607 nm (Figure 13). The absorbance band increase linearly depending on oxalate concentrations from 1.76 to 49.4 μM with a limitation of detion of 0.62 μM.

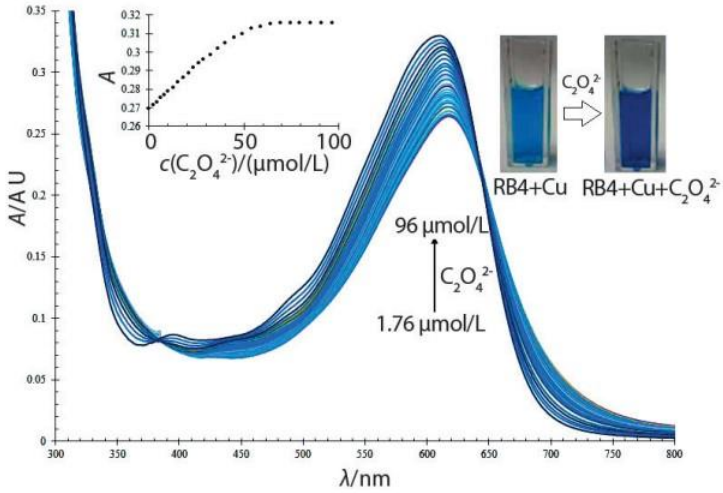

Figure 13. IDA for oxalate sensing by RB4-Cu^{2+}.

Left; the UV-Vis absorption of the RB4-Cu^{2+} complex changes (increases) as a function of the concentration of oxalate. Right; the photograph of the solution color change from sky blue to dark blue after adding oxalate [49]

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Conceptual framework

The supersaturation of lithogenic ions, especially oxalate, in urine is the key role to drive crystal formation. Excessive oxalate and crystal formed in urine causing renal cell injury, crystal retention and kidney stone formation. Therefore, a high level of urinary oxalate can be a marker for assessing risk of CaOx stone formation. Since, level of urinary oxalate has a clinical importance, we were interested to develop a new oxalate measurement technique for detecting oxalate in urine based on non-enzymatic assay and enzymatic assay. For non-enzymatic assay, oxalate measurement was developed based on the principle of indicator displacement assay (IDA) using a new chemical complex (Cu-nat5-BPG) that was proposed to be highly selective for oxalate. In the enzymatic assay, the oxalate measurement was developed by using oxalate oxidase enzyme (OxOx) couple with horse radish peroxidase enzyme (HRP). Since the commercial oxalate oxidase enzyme was expensive, we aimed to produce the recombinant barley-oxalate oxidase protein in yeast system (*Pichia pastoris*) (Figure 14).

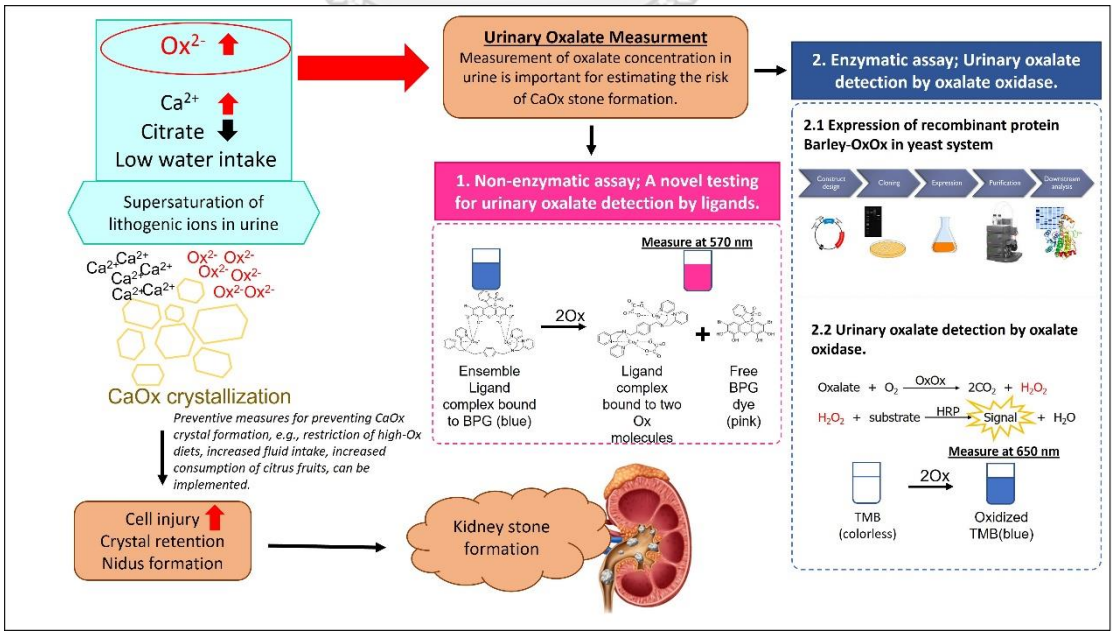

Figure 14. Conceptual framework of this study

Chapter 3

Materials and Methods

Study design

The study was a cross-sectional observational analytical study to measure oxalate content in human urine samples from stone-forming and non-stone forming subjects by a newly developed IDA test based on oxalate-binding ligands and the oxalate oxidase enzymatic method.

Urine specimen collection

In this study, we used the human urine specimens from the our previous project entitled: 'Test accuracy of urinary calcium oxalate crystallization index (COCI) for diagnosis of urolithiasis, and development of an innovative quantum dot nanoparticle-based method for determination of urinary oxalate' (IRB:286/59) with the consent from the subjects and approval from the ethic committee of Faculty of Medicine, Chulalongkorn University. The 24-hour urine samples were collected from the healthy volunteers (non-stone forming subjects) and kidney stone patients admitted to Mahasarakham province during 2017 and 2018. All of participants in the research project (IRB:286/59) had signed a consent form after being informed for all of information of the research project, including the potential risks. The subjects were divided into two groups, non-stone forming control group (n=121) and kidney stone group (n=69). Inclusion and exclusion criteria are shown below.

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Inclusion criteria

- Non-stone group; males and females aged 18 years and over.

- Kidney stone group; patients with kidney stones aged 18 years and over admitted to Mahasarakham Hospital for stone treatment.

Exclusion criteria

- Non-stone group; subjects with urinary tract infections or having a history of kidney stone.

- Kidney stone group; patients with hematuria, using drugs that cause urine to change color or having urinary tract infection or any malignancies.

Sample preparation

The collected urine samples from healthy volunteers and kidney stone patients were thawed (from -20 ºC) for oxalate detection. The samples were filtrated and pretreatment with and without activated charcoal (25 mg/mL). For samples treated with activated charcoal, samples were centrifuged at 10,000 xg for 5 min and the supernatant was collected. The samples were diluted to 20% in the buffer pH 7.4 to minimize the interfering effects from interferences. The samples were stored at -20 ºC before testing.

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Indicator displacement assay (IDA) for selective sensing of oxalate

Preparation of sensing ensemble; add BPG indicator solution (400 μM, 10 μL) in 10 mM HEPES buffer, pH 7.4 (prepared 80:20 acetonenitrile:water) to Cu-nat5 ligand complex solution (20 μM, 200 μL, prepared in the same solvent system). 40 μL of chemicals to be tested for non-specific binding (Table 2), diluted artificial urine to be used for mimicking the real urine with and without oxalate for establishing standard curve (Table 3) or each diluted urine sample were added to the sensing ensemble solution. The absorbance was measured at 570 nm (a maximum absorption wavelength

of BPG indicator). Sodium oxalate at various concentrations was used as standards to generate the standard curve. Concentration of oxalate was calculated from the generated standard curve.

Table 2. List of chemicals tested for non-specific binding to the ligands.

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Target substances (common in urine)	Chemical used, tested concentration	Target substances (other chemicals)	Chemical used, tested concentration
Ammonia	Ammonia, 30mM	Acetate	Sodium acetate, 1mM
Calcium	Calcium chloride dihydrate, 3mM	Carbonate	Sodium carbonate, 1mM
Citrate	Sodium citrate dihydrate, 4mM	Cyanide	Sodium cyanide, 1mM
Citric acid	Citric acid monohydrate, 4mM	Fluoride	Sodium fluoride, 1mM
Creatinine	Creatinine, 8mM	Formate	Sodium formate, 1mM
Magnesium	Magnesium chloride, 4mM	Fumarate	Disodium fumarate, 1mM
Phosphate	Disodium hydrogen phosphate, 25mM	Glutarate	Disodium glutarate, 1mM
Potassium	Potassium chloride, 40mM	Glycolate	Sodium glycolate, 1mM
Pyrophosphate	Sodium pyrophosphate tetrabasic, 1mM	Hydrogen carbonate	Sodium hydrogen carbonate, 1mM
Sodium and chloride	Sodium chloride, 170mM	Hydroxide	Sodium hydroxide, 1mM
Sulfur	Sodium sulfate anh., 55mM	Iodide	Sodium iodide, 1mM
Urea	Urea, 330mM	Lactate	Sodium DL-lactate, 1mM
Uric acid	Uric acid, 1.8mM	Malate	Sodium DL-malate hydrate, 1mM
		Nitrate	Sodium nitrate, 1mM
		Succinate	Disodium succinate, 1mM
		Tartrate	Disodium tartrate dihydrate, 1mM

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Table 3. The formula of artificial urine modified from AU-siriraj (S. Chutipongtanate and V. Thongboonkerd, 2010) without oxalate for establishing standard curve [50] as shown in Table 3.

Property and composition	Chemical used	tested concentration
pH	-	6.20
Specific gravity	-	1.01 g/ml
Osmolality	-	446 mOsm/kg
Urea	Urea	200 mM
Uric acid	Uric acid	1.00 mM
Creatinine	Creatinine	4.00 mM
Sodium citrate	Sodium citrate dihydrate	5.00 mM
NaCl	Sodium chloride	54.00 mM
KCl	Potassium chloride	30.00 mM
NH_4Cl	Ammonium chloride	15.00 mM
$CaCl_2$	Calcium chloride dihydrate	3.00 mM
$MgSO_4$	Magnesium sulfate heptahydrate	2.00 mM
$NaHCO_3$	Sodium carbonate	2.00 mM
Na_2SO_4	Sodium sulfate anh.	9.00 mM
NaH_2PO_4	Sodium dihydrogen phosphate monohydrate.	3.60 mM
Na_2HPO_4	Disodium hydrogen phosphate anh.	0.40 mM

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Target OxOx gene selection

We selected the gene for recombinant barley OxOx expression based on the publication of Whittaker et al. reported in 2002 (also put Ref. here), barley germin mRNA, partial cds(GenBank L15737 (previous GenBank 289356)) (Fig.15). The sequence was the complete mature peptide sequence with codon usage optimized for *P. pastoris* expression.

Barley germin mRNA, partial cds

GenBank: L15737.1

FASTA Graphics

Go to:

```
LOCUS       BLYGERMIN               603 bp    mRNA    linear   PLN 03-AUG-1993
DEFINITION  Barley germin mRNA, partial cds.
ACCESSION   L15737
VERSION     L15737.1
KEYWORDS    germin; oxalate oxidase.
SOURCE      Hordeum vulgare
  ORGANISM  Hordeum vulgare
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliopsida; Liliopsida; Poales; Poaceae; BOP
            clade; Pooideae; Triticodae; Triticeae; Hordeinae; Hordeum.
REFERENCE   1  (bases 1 to 603)
  AUTHORS   Lane,B.G., Dunwell,J.M., Ray,J.A., Schmitt,M.R. and Cuming,A.C.
  TITLE     Germin, a protein marker of early plant development, is an oxalate
            oxidase
  JOURNAL   J. Biol. Chem. 268 (17), 12239-12242 (1993)
   PUBMED   8509360
COMMENT     Original source text: Hordeum vulgare cDNA to mRNA.
FEATURES             Location/Qualifiers
     source          1..603
                     /organism="Hordeum vulgare"
                     /mol_type="mRNA"
                     /db_xref="taxon:4513"
     CDS             <1..>603
                     /EC_number="1.2.3.4"
                     /note="This is the sequence of the complete mature
                     peptide."
                     /codon_start=1
                     /product="oxalate oxidase"
                     /protein id="AAA32959.1"
                     /translation="SDPDPLQDFCVADLDGKAVSVNGHTCKPMSEAGDDFLFSSKLTK
                     AGNTSTPNGSAVTELDVAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPRATEIGMVMKG
                     ELLVGILGSLDSGNKLYSRVVRAGETFVIPRGLMHFQFNVGKTEAYMVVSFNSQNPGI
                     VFVPLTLFGSDPPIPTPVLTKALRVEAGVVELLKSKFAGGS"
ORIGIN
        1 tccgacccag acccactcca ggacttctgc gtcgcggacc tcgatggcaa ggcggtctcg
       61 gtgaacgggc atacgtgtaa gcccatgtcg gaggccggcg acgacttcct cttctcgtcc
      121 aagctgacca aggccggcaa cacgtccacc ccgaacggct cggccgtgac ggagctcgac
      181 gtggccgagt ggcccggtac gaacacgctg ggcgtgtcca tgaaccgtgt ggacttcgcg
      241 ccgggggcca ccaacccgcc gcacatccac ccgcgtgcaa ccgagatcgg catggtgatg
      301 aaaggtgagc tcctcgttgg aatcctcggc agccttgact ccggaaacaa gctctactcc
      361 agggtggtgc gtgccggaga gactttcgtc atcccgcgcg gcctcatgca cttccagttc
      421 aacgttggta agacggaagc ctacatggtt gtgtccttca acagccagaa ccctggcatc
      481 gtcttcgtgc cgctcacact cttcggctcc gaccctccca tccccacgcc cgtgctcacc
      541 aaggctctcc gggtggaggc cggagtcgtg gaacttctca agtccaagtt cgccggtggg
      601 tct
```

Figure 15. Barley germin mRNA, partial cds, GenBank accession number L15737

Information from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/289356)

Recombinant gene design

The barley oxalate oxidase (barley OxOx) gene was cloned into *P. pastoris* expression vector pPICZαA (Invitrogen, USA) located next to the sequence encoding for alpha factor (secretory signal sequence) (Fig. 16). The cloning strategy is shown in pPICZαA expression vector map in Fig. 17. The synthesized (or inserted?) gene was consisting of 5 components including *Xho I* restriction sequence, residual sequence of alpha factor, target sequence (barley OxOx gene), stop codon, and *Not I* restriction sequence. The total size was 645 bp as shown in Fig. 16 below. The synthesized gene was then delivered into the pUC57 vector (Fig. 18). The synthesized gene in pUC57 vector was cut with *Xho I* and *Not I* and then ligate it into the expression vector pPICZαA.

Sequence of the synthesized gene, 645 bp:

Xho I | Distal end of alpha factor | Barley OxOx

5'GGCTCGAGAAAAGAGAGGCTGAAGCTTCTGACCCTGACCCACTTCAAGATT
TCTGCGTCGCTGATTTGGATGGTAAAGCAGTCTCCGTTAATGGACACACCTGT
AAACCTATGTCTGAAGCTGGAGATGATTTCTTGTTTTCTTCTAAGTTGACTAAAG
CTGGTAACACTTCTACTCCAAATGGTTCTGCTGTTACTGAATTGGATGTTGCTG
AGTGGCCTGGTACTAACACTTTGGGTGTTTCTATGAATAGAGTTGATTTTGCTC
CAGGTGGTACTAACCCACCTCATATTCACCCTAGAGCTACTGAAATTGGTATG
GTTATGAAGGGAGAGTTGTTGGTTGGTATTTTGGGTTCTTTGGATTCTGGTAAC
AAATTGTACTCCAGAGTTGTTAGAGCTGGTGAAACTTTTGTTATTCCAAGAGGT
TTGATGCATTTCCAATTCAATGTTGGTAAAACTGAGGCTTACATGGTTGTTTCTT
TCAACTCTCAAAATCCAGGTATTGTTTTTGTTCCTTTGACTTTGTTCGGTTCTGAT
CCACCTATTCCAACTCCTGTTTTGACTAAAGCTTTGAGGTTGAGGCTGGAGTTG
TTGAGTTGTTGAAGAGTAAGTTCGCTGGTGGAAGTTAATAAGCGGCCGCCC 3'

Stop codon | Not I

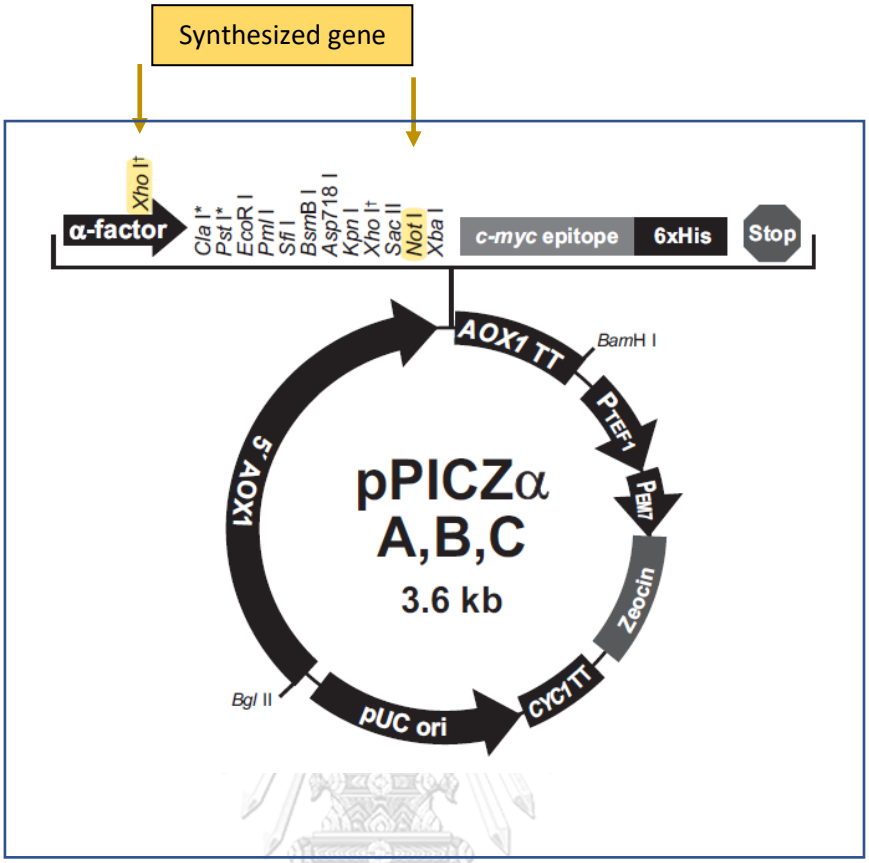

Figure 16. pPICZαA expression vector map

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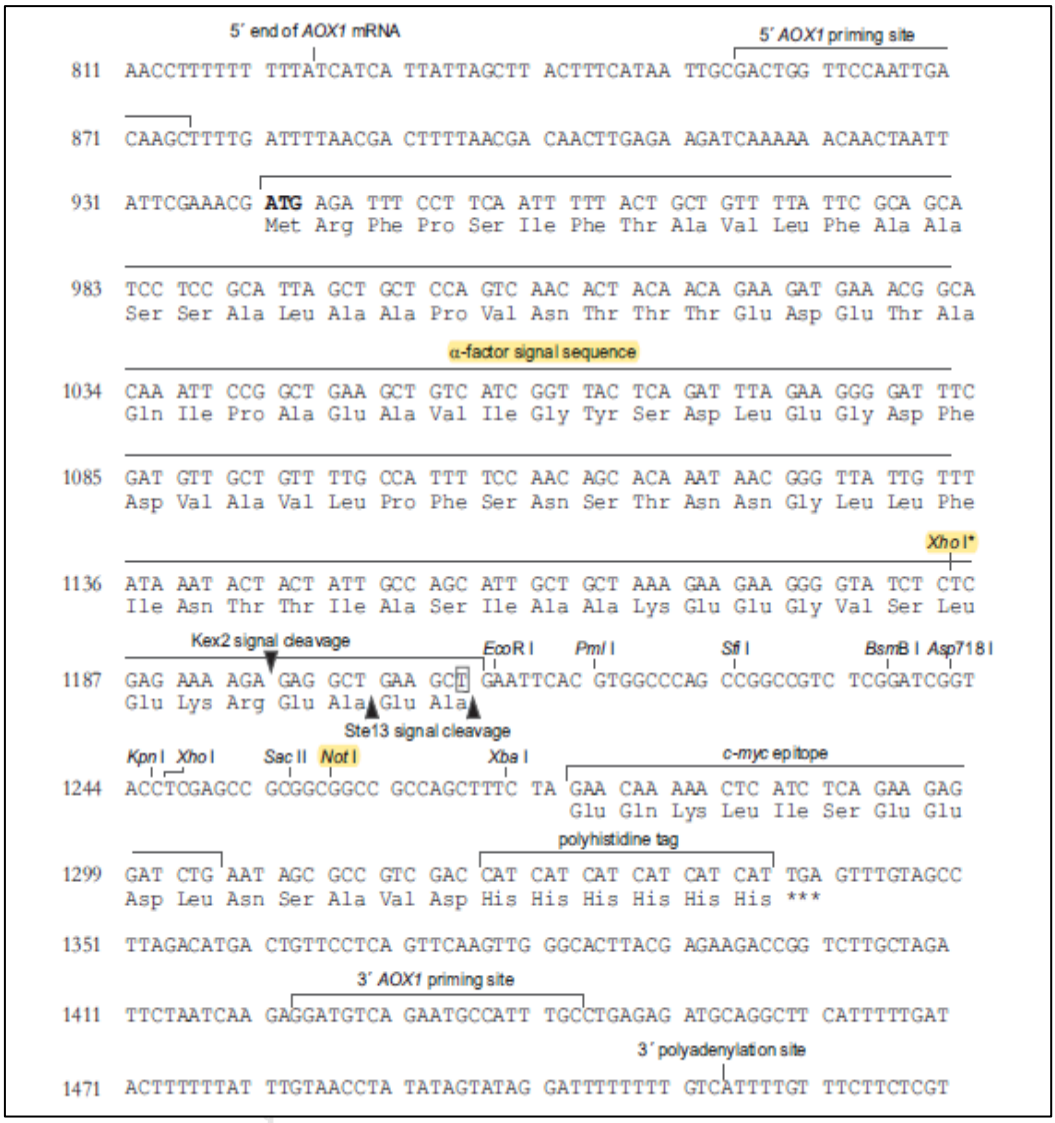

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Figure 17. The sequence of alpha leader sequence with multiple cloning sites in pPICZαA

Information from Cat. no. V195-20 Rev. Date: 7 July 2010 Manual part no. 25-0150

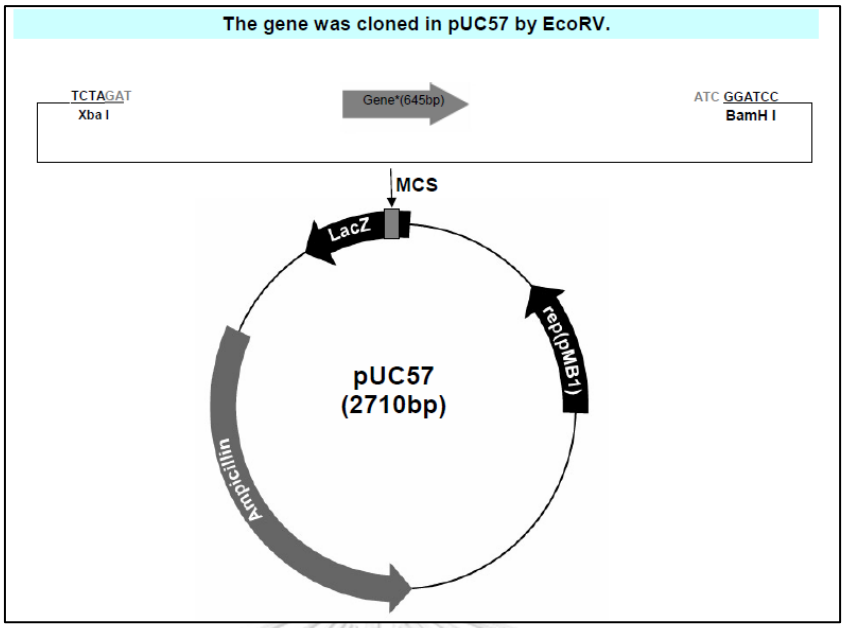

Figure 18. pUC57 expression vector map

E. coli competent cell preparation

E. coli in glycerol stock was inoculated into full name (LB) media and incubated for 12-16 h at 37°C under shaking condition (200 rpm). Cell growth was measured by absorbance (OD_{600} nm). The cells were re-cultured (started at OD_{600} nm = 0.01) by incubating at 37°C under shaking condition 200 rpm until the cell density reached OD_{600} nm = 1.0-1.2. The cells were centrifuged at 4,000 rpm for 5 min and washed 3 times with 100 mM $CaCl_2$. The cells were suspended in 500 µL of 100 mM $CaCl_2$ and pre-chilled on ice or keep cool for transformation. This cell suspension was called *E. coli* competent cells.

E. coli transformation

Transformation and propagation of the plasmids (vectors) (pUC57-barley-OxOx, pPICZαA-Derp8 and pPICZαA-barley-OxOx) in *E. coli*. The E. coli competent cells (100 µL) were mixed with 30 ng of plasmid in a tube. Negative control

was also prepared by mixing *E. coli* competent cells (100 µL) with the same volume of sterile water. Tubes were pre-chilled on ice for 12-15 min. For heat shocking, the tubes were incubated in water bath at 42°C for 90 sec and the placed them immediately on ice for 10 min. 800 µL of super optimal broth with catabolite repression (SOC) media was added and incubated at 37°C under shaking condition 200 rpm for 1-2 h. After that, cells were centrifuged at 6,000 rpm for 2 min and spreaded onto LB agar with and without antibiotics. Antibiotics selection was based on drug resistance genes presented in each vector. The pUC57 vector used Amplicilin 100 µg/mL while pPICZαA used Zeozin 100 µg/mL for selection. The agar was incubated for 14-16 h at 37°C. The colonies appeared on LB agar with antibiotics indicated a successful transformation process.

Propagation of the plasmids in transformed *E. coli*

Several colonies from LB agar containing antibiotics were picked and inoculated into LB media with the same antibiotics and then incubated for 12-16 h at 37°C under shaking condition 200 rpm. The obtained number of plasmids increased correspondingly to the number of transformed *E. coli*.

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Vector extraction

Presto™ Mini Plasmid kit (Geneaid, Taiwan) used to extract and purify any plasmid DNA from the cultured transformed *E. coli*. The plasmid DNA extraction was performed according to the manufacturer's protocol. The purified plasmid DNA was quantified at A_{260nm}/A_{280nm} by NanoDrop™ 2000/2000c Spectrophotometers (Thermofisher Scientific, USA) and kept at -20°C.

Restriction enzyme digestion

The linear synthesized or target gene (barley-OxOx) and linear pPICZαA gene were excised from pUC57-barley-OxOx and pPICZαA-Derp8 by *Xho I* and *Not I* digestion (Fig 19). The restriction sites of *Xho I* and *Not I* are shown in Fig. 20 and Fig. 21, respectively. The DNA vector digestion was performed according to the manufacturer's protocol (BioLabs, USA). In brief, DNA vector barley-OxOx or pPICZαA-Derp8, restriction enzyme and 10X NE buffer were mixed in the microcentrifuge tube. The tube was incubated at 37°C for 1-3 h, then at 65°C for 20 min for enzyme inactivation. Both synthesized gene (barley-OxOx) and pPICZαA gene were digested by Xho I and Not I at the same time. The digested DNA were separated by agarose gel electrophoresis and purified by DNA extraction.

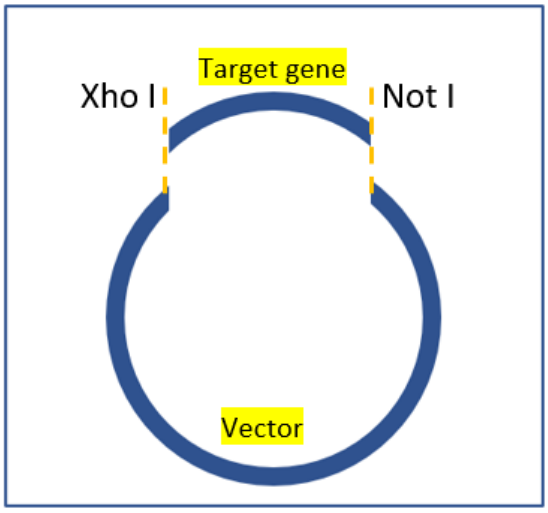

Figure 19. The restriction enzymes double digest for the target DNA

Figure 20. The restriction site of Xho I enzyme

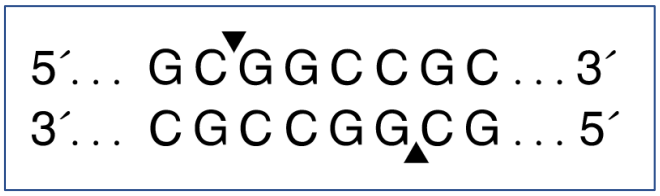

Figure 21. The restriction site of Not I enzyme

Agarose gel electrophoresis and DNA extraction

The digested DNA products were mixed with loading dye (Invitrogen, USA) and run on 1% agarose gel electrophoresis using TAE as a buffer. 5 µL of 1kb DNA ladder (Invitrogen, USA) was served as a standard DNA. The synthesized DNA products and pPICZαA plasmids were checked by agarose gel electrophoresis and visualized under UV light exposure by using Gel-DOC XR (Bio-rad, USA)

Gel extraction

QIAquick gel extraction kit (QIAGEN, Germany) was used to extract DNA products from the agarose gel. The target DNA from the agarose gel was excised with a clean and sharp blade. The DNA extraction was performed according to the manufacturer's protocol. The purified plasmid DNA was quantified at A_{260nm}/A_{280nm} by NanoDrop™ 2000/2000c Spectrophotometers (Thermofisher Scientific, USA) and kept at -20°C.

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Vectors ligation

The purified synthesized gene (barley-OxOx) and purified pPICZαA gene were ligated to be a vector pPICZαA-barley OxOx by T4 ligase enzyme (BioLabs, USA). The DNA vector ligation was performed according to the manufacturer's protocol. In brief, the insert DNA (barley-OxOx), DNA vector (pPICZαA), enzyme T4 ligase and T4

buffer were mixed in the microcentrifuge tube. The tube was incubated at 16°C for 14-16 h. Then the enzyme was inactivated at 65°C for 10 min.

P. pastoris competent cell preparation

P. pastoris X33 strain in glycerol stock was inoculated into YPD media and incubated at 30°C for 14-16 h under shaking condition (250 rpm). Cell growth was measured by optical absorption at OD_{600} nm. Cells were re-cultured at the starting concentration of OD_{600} nm = 0.2 in 200 mL of YPD media. Cells were incubated at 30°C under shaking condition 250 rpm until the cell density reached OD_{600} nm = 1.3-1.5. After centrifugation (4,000 rpm for 5 min, 4 °C), cells were washed 3 times with ice-cold sterilized water. Cells were suspended in 8 mL of fresh solution of 100 mM LiAC, 10 mM DTT, 0.6 M sorbitol and 10 mM Tris-HCl pH7.5, and then pre-chilled on ice for 30 min. After centrifugation, cells were washed again 3 times with ice-cold 1 M sorbitol. The cells were suspended in 1 mL of 1M sorbitol. The cells were kept on ice for further transformation.

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P. pastoris transformation by electroporation

Transformation of the target linearized plasmids, pPICZαA-barley-OxOx vector, into *P. pastoris* was started from linearization of the plasmid with Sac I restriction enzyme (NEB, USA). Then, the linearized pPICZαA-barley-OxOx was purified by QIAquick gel extraction kit. Purified linearized pPICZαA-barley-OxOx (5 mg) was transferred to an ice-cold 2 mm electrode cuvette and incubated with the *P. pastoris* competent cells for 5 min. The Gene-PulserXcell™ (Bio-rad, USA) was used to transform the plasmid at 1500 V, 200 Ω in the electrode cuvette. The cells were transferred to a new tube, mixed with 1 M sorbital in YPD media and incubated at 30°C for 1-2 h. After that, cells were spined down and plated on the full name (YPDS) agar (YPD with 1 M

sorbitol agar) with and without the Zeocin 100 μg/mL. The agar was incubated at 30°C for 2-7 days. The colonies grown on YPDS agar containing antibiotics indicated success of transformation.

Barley-OxOx expression

The positive Zeocin-resistant colonies were selected, inoculated into YPD media, and incubated overnight (14-16 h) at 30°C under shaking condition (as the first culture). The cells were transferred into BMGY medium as the second culture under the same culture condition. The cells were harvested by centrifugation and washed twice with full name (BMxY) medium. Then the cells (at a final concentration of $OD_{600\ nm}$ = 10) were transferred into BMMY medium containing 0.5% methanol to induce barley-OxOx expression. Every 24 h, the culture medium was collected, and methanol was re-added to maintain the inducer concentration. The soluble protein from the supernatant of cells was analyzed by SDS-PAGE.

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SDS-PAGE

SDS-PAGE (15% resolving gel and 4% stacking gel) was performed for detection of expressed barley-OxOx protein. The supernatant sample was mixed with 4X loading dye with reducing reagent (ratio 3:1) and heated at 95 °C before loading. PageRuler™ Prestained 5 μL of protein ladder 10-180 kDa (Thermofisher Scientific, USA) was used as a protein marker. After electrophoresis (180 V for 60-80 mins) the gel was stained with Coomassie blue R-250 (Bio-rad, USA) overnight and destained by destaining solution (10% acetic acid, 40% methanol and 50% distilled water (V/V)).

Barley-OxOx purification by ion-exchange chromatography

For the first step of purification, the supernatant containing secreted barley-OxOx was diluted ten times with MilliQ water, and pH was adjusted to pH 4. This diluted solution was applied onto a CM Sepharose ion-exchange chromatography column (GE Healthcare, UK) that connected with AKTA prime purification system (GE Healthcare, UK). Then the column was equilibrated with 50 mM glycine-HCl (pH 3) at the flow rate of 10 mL/min. The unbound protein was flushed from the column with the equilibrating buffer until the absorbance at A_{280} nm reached the baseline level. The stepwise elution was performed using 50 mM glycine-HCl (pH 3) containing different concentrations at 100 mM, 200 mM, 300 mM, 500 mM, and 1000 mM NaCl. The purified fractions were analyzed by the SDS-PAGE.

Size exclusion chromatography

For the second step of purification, the purified fractions of barley-OxOx from ion-exchange chromatography were pooled together and concentrated using 10 kDa cut-off membrane (GE Healthcare, UK). The barley-OxOx was subjected onto a Superdex 75 HR column (GE Healthcare, UK) and eluted at a flow rate of 0.5 mL/min in PBS buffer pH 7.4. The final purified fractions were analyzed by the SDS-PAGE.

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In-gel digestion for mass spectrometry

Protein samples were separated by SDS-PAGE and stained with Coomassie blue R-250 (Bio-rad, USA) for 5-15 min (until seeing the margins of the band to be able to cut, not too long because it took a long time for destaining.). The gel was destained by water-optimized LC/MS at room temperature with gentle agitation for 1 h. The target protein band was cut into slices by a new blade and wipe it with ethanol before using it.

The cut gel was chopped into small cubes (1-2 mm^2) and placed into 1.5 mL tube. Then, 100-300 µL of 25mM NH_4HCO_3 in 50% can was added, vortexed, briefly centrifuged and stood for 10 min (repeated twice). The supernatant was discarded. The gel pieces shrank and became white. The gel pieces were spined for 10-20 min until it was completely dried by SpeedVac. The white gel was sent to the laboratory of Prof. Dr. Trirak Pisitkun for the next steps of protein identification be mass spectrometry, reduction and alkylation, enzyme digestion, extraction of peptides, zip tip protocol for peptide and protein analysis.

Enzymatic activity testing

The activity of recombinant barley-OxOx from purified fraction was determined by enzymatic assay. The reaction reagent was prepared by mixing 160 µL of working full mane (TMB) in succinate buffer pH 3.8, 30 µL of 0.1 M oxalic acid and 2 µL of 0.04 mg/mL full name (HRP), and 30 µL of the purified fraction sample. The reaction was performed in 96-well plate and incubated at room temperature for 30 minutes in the dark. Then the reaction was stopped by 50 µL of 0.16 M H_2SO_4. The absorbance at 450 nm was measured using a microplate reader (Thermo Scientific, USA).

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Urinary oxalate measurement by enzymatic assay using commercial OxOx enzyme

The levels of soluble oxalate in urine samples were measured by OxOx (purchased from the Roche diagnostic, Germany) and HRP (Sigma, USA) enzymatic assay. The urine sample was pretreated by activated charcoal (Sigma, USA) using 80 mg of charcoal with 1 mL of the urine sample incubated at room temperature for 10 min with interval mixing. Then samples were centrifuged at 10,000 xg for 5 min, and

supernatant was collected for the assay. First, the supernatant (20 µL) was transferred into 6 separated wells: 2 wells for sample blank, 2 wells for sample test, and 2 wells for oxalate spike (internal standard). Second, type I water (5 µL) was added to sample blank wells and sample test wells, and 5 µL of 1.25 mM sodium oxalate was added to the oxalate spike wells. Third, the reaction reagent containing 110 µL of working TMB (0.1 mg/mL) in succinate buffer pH 3.8, 40 µL of 2 mg/ml OxOx enzyme, and 1 µL of 2 mg/ml HRP enzyme. The reagent mixed for the sample blank (without OxOx) was prepared by mixing 110 µL of working TMB in succinate buffer pH 3.8, 40 µL of distilled water, and 1 µL of 2 mg/ml HRP enzyme. The reaction was performed in 96-well plate and incubated at room temperature for 10 min in the dark. The absorbance at 650 nm was measured using a microplate reader (Thermo Scientific, USA). Urinary oxalate was calculated according to the formula shown below.

$$\text{Urinary oxalate (µM)} = \frac{(\text{OD sample test} - \text{OD sample blank})}{(\text{OD oxalate spike} - \text{OD sample test})} \times 250 \times 1.25$$

$$\text{Urinary oxalate (mg/day)} = \frac{\text{urinary oxalate (µM)}}{1000} \times 88.02\ (molecular\ weight\ of\ oxalate)\ x\ volume\ of\ urine\ per\ day\ (L)$$

NOTE:

- OD_{SAMPLE}, OD_{SPIKED}, and OD_{BLANK} were average absrobance of the sample test, oxalate spike, and sample blank wells, respectively.
- 250 µM is the effective concentration of the spiked oxlate
- 1.25 is the dilution factor.

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Statistical analysis

All data were presented as mean ± standard deviation (SD) or median ± interquartile range as appropriate. Two-sample paired-sample t-test or Wilcoxon signed-rank test was used for comparing the mean of urinary oxalate level between the newly developed method and the standard oxalate oxidase method. Cohen's kappa test was used to assess agreement and reliability between newly method and oxalate oxidase medthod [51]. Also, Pearson's correlation test or Spearman rank correlation test was tested to find the correlation between the two tests. All graphs and statistics will be created and computed by GraphPad Prism Software version 6.0. Significant difference is set at *p-value* < 0.05.

Ethical consideration

A part of this research was performed in human urine samples. Therefore, the research protocol was reviewed and approved by the Institutional Ethics Committees, Faculty of Medicine, Chulalongkorn University. (IRB419-62)

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Chapter 4

Result

Part1: Cu-Nat5-BPG ligand specific oxalate

1.1 Specificity of Cu-Nat5-BPG system with anion substances (5mM) in 80% acetonitrile aqueous solution buffered with 10 mM HEPES pH 7.0.

The addition of Cu-Nat5 to BPG solution in 80% acetonitrile aqueous solution buffered with 10 mM HEPES pH 7.0 resulting in the color change from pink color of free BPG to blue color of ensemble (Cu-Nat5-BPG). Then, this Cu-Nat5-BPG ensemble was treated with several interested anions in the same solvent system. The result showed that oxalate and cyanide could turn the blue color of the ensemble to pink color of free BPG, indicated that the BPG was replaced by oxalate and cyanide. (Fig. 22 and Fig.23)

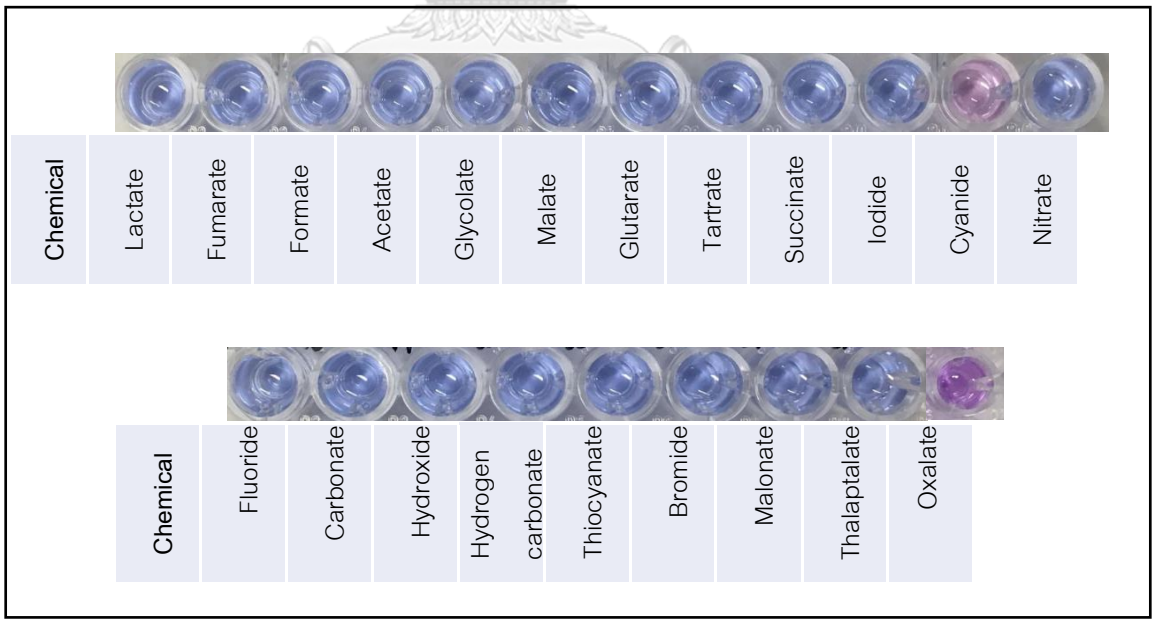


Figure 22. The naked eye results of the specificity of Cu-Nat5-BPG system with anion substances (5mM)

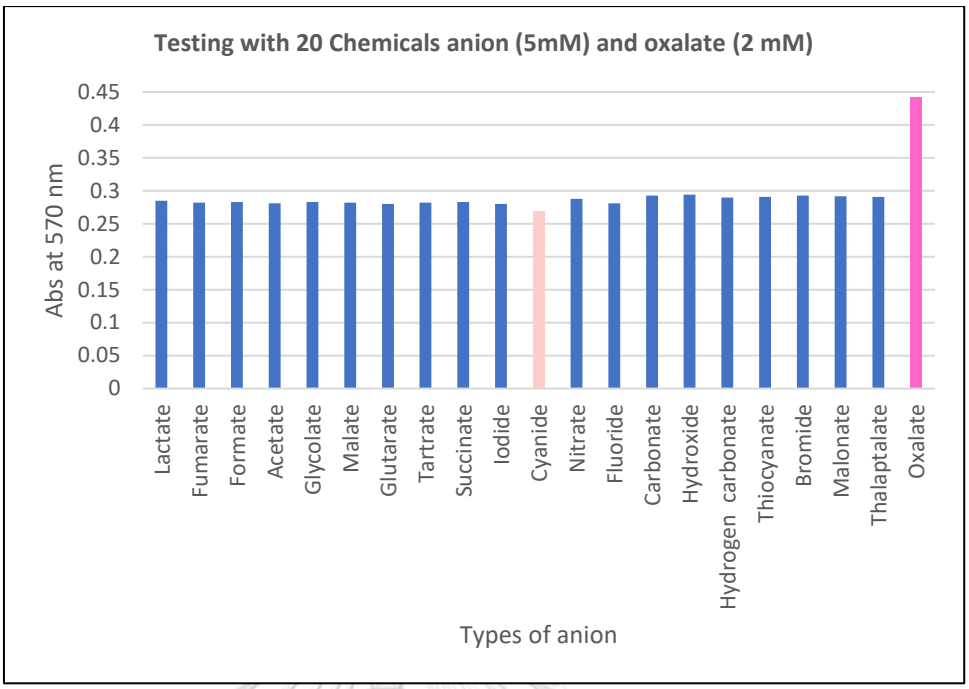
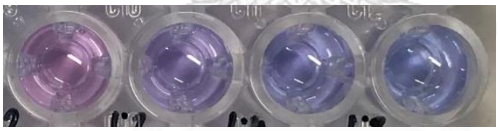
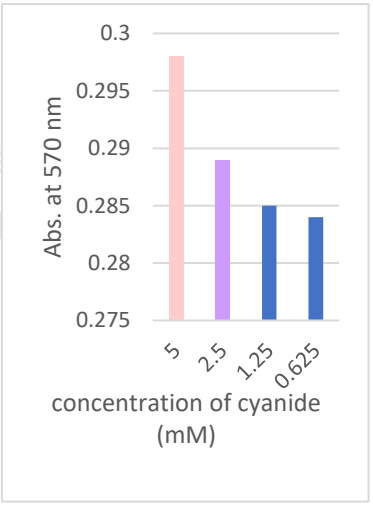


Figure 23. The absorbance of the Cu-Nat5-BPG system with anion substances (5mM)

1.1.1 Cyanide serial dilution test



Chemical name	Sodium Cyanide			
Dilution factor	1	1:2	1:4	1:8
Conc. (mM)	5	2.5	1.25	0.625



At diluted concentrations of 2.5-5 mM reaction with sodium cyanide revealed a positive reaction (Absorbance at 570nm was slightly changed compared with the blank control, but the color was obviously changed.). It was expected that cyanide would have a less interfering effect in urine samples because normally the concentration of cyanide

in urine is very low (211-641 nM). According to literature, urinary cyanide in non-smoker and smokers are 217±6 nM and 518±123 nM, respectively [52].

1.2 Reactivity of common urinary substances to the Cu-Nat5-BPG ensemble in 80% acetonitrile aqueous solution buffered with 10 mM HEPES pH 7.0

We tested the specificity of the Cu-Nat5-BPG ensemble with other substances that were commonly presented in the urine. The result found that creatinine (17.67 mM) and PPi (25 mM) could also bind to the ensemble. Creatinine clearly changed the color of the solution from blue to pink (Fig.24), and it also changed the maximum absorption of the solution (Fig. 25). The exact mechanism is still unknown. This phenomenon was also found in the case of PPi.

Chem. name	Urea	NaCl	KCl	Creatinine	Na_2SO_4	Pi	PPi
Conc.(mM)	330	170	40	17.68	55	25	25

Chem. name	Citric A	NaCitrate	Amm	Uric A	$CaCl_2$	$MgCl_2$	Oxalate
Conc.(mM)	4	4	30	1.8	3	4	2

Figure 24. The naked eye results of the specificity of Cu-Nat5-BPG system with compositions in urine

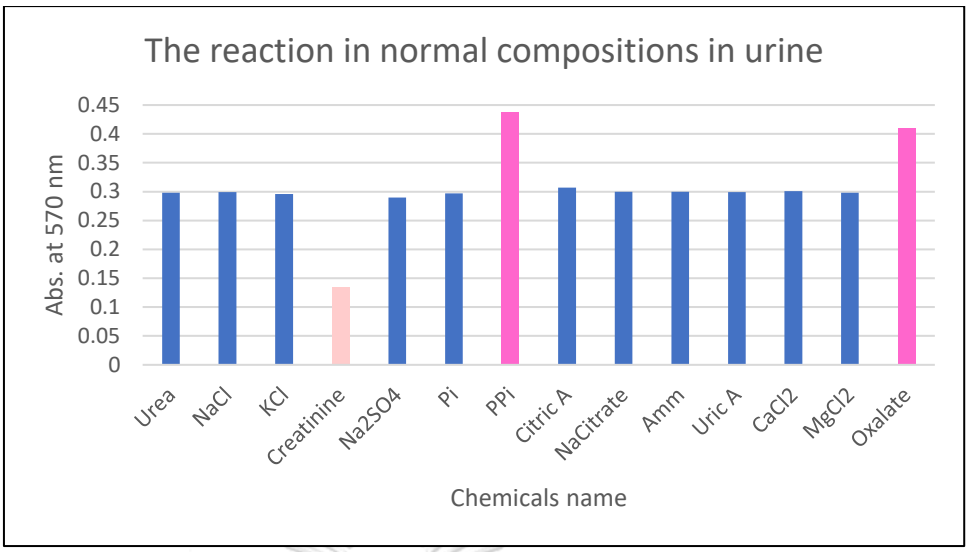


Figure 25. The absorbance of the Cu-Nat5-BPG system with common urinary substances

1.2.1 Creatinine serial dilution test

At diluted concentrations of 4.42-8.84 mM reaction with creatinine revealed a positive reaction and changed the maximum absorption of the solution. It was expected that creatinine would have an importance interfering effect in urine samples because the concentration of creatinine in urine is in range 600-3000 mg/day or 3-22 mM. According to the reference of the Mayo Clinic.

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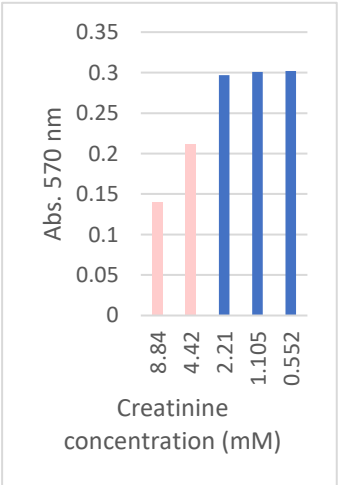
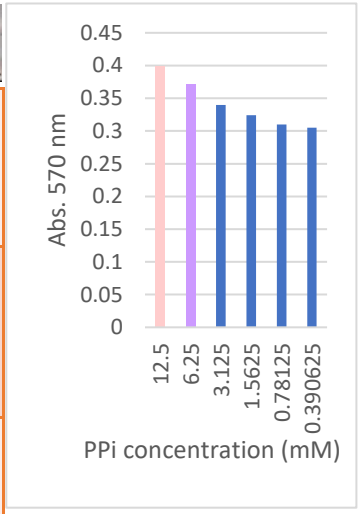
Chemical name	Creatinine				
Dilution factor	1:2	1:4	1:8	1:16	1:32
Conc. (mM)	8.84	4.42	2.21	1.105	0.552
Average absorbance (570nm)	0.140	0.212	0.297	0.301	0.302



1.2.2 Pyrophosphate (PPi) serial dilution test

At diluted concentrations of 6.25-12.5 mM reaction with PPi revealed a positive reaction (at concentration 6.25 mM the absorbance at 570nm was slightly changed compared with the blank control, but the color was obviously changed.). It was expected that PPi would have a less interfering effect in urine samples because normally the concentration of PPi in urine is very low. According to literature, urinary pyrophosphate in normal subjects and in stone formers are 1-10 mg/day or 0.5-8 μM [53].



Chemical name	PPi					
Dilution factor	1:2	1:4	1:8	1:16	1:32	1:64
Conc.(mM)	12.5	6.25	3.125	1.563	0.781	0.391
Average absorbance (570nm)	0.399	0.372	0.340	0.324	0.310	0.305

1.3 Selectivity of oxalate to Cu-Nat5-BPG ensemble in artificial urine

To determine whether Cu-Nat5-BPG could be a specific and effective method for measuring oxalate, we performed the oxalate and ensemble reaction in the presence of various urinary substances in 80% acetonitrile aqueous solution buffered with 10 mM HEPES pH 7.0. The tested concentrations of urinary substances were based on their concentrations in normal human urine (Fig. 26). Compared with reaction of Cu-Nat5-BPG ensemble and oxalate without other urinary substances, the result showed that $CaCl_2$ (%absorbance = 74.9%) and $MgSO_4$ (%absorbance = 73.3%) (Fig. 27) remarkedly change the absorbance at 570 nm, indicated that $CaCl_2$ and $MgSO_4$ interfered or quenched the reaction of oxalate and ensemble. Furthermore, the color of the solution instead of changing to pink due to the oxalate replacement, it was converted to blue of Cu-Nat5-BPG. The data suggested that cations decreased the selectivity of oxalate to the Cu-Nat5-BPG ensemble. Serial dilution of five cations (Ca^{2+}, Mg^{2+}, Na^+ and K^+ and NH_4^+) were performed to test if their interfering effects were decreased when their concentrations were decreased. As expected, the result demonstrated that the lesser concentrations caused lesser interfering effect (Fig. 28).

Property and composition	AU-Siriraj (this study)
pH	6.2
Specific gravity (g/ml)	1.010
Osmolality (mOsm/kg)	446
Urea (mM)	200
Uric acid (mM)	1.00
Creatinine (mM)	4.00
$Na_3C_6H_5O_7$ (mM)	5.00
NaCl (mM)	54.00
KCl (mM)	30.00
NH_4Cl (mM)	15.00
$CaCl_2$ (mM)	3.00
$MgSO_4$ (mM)	2.00
$NaHCO_3$ (mM)	2.00
$Na_2C_2O_4$ (mM)	0.10
Na_2SO_4 (mM)	9.00
NaH_2PO_4 (mM)	3.60
Na_2HPO_4 (mM)	0.40
Purpose	Multipurpose

Figure 26. The formula of artificial urine modified from AU-siriraj

(S. Chutipongtanate and V. Thongboonkerd, 2010)

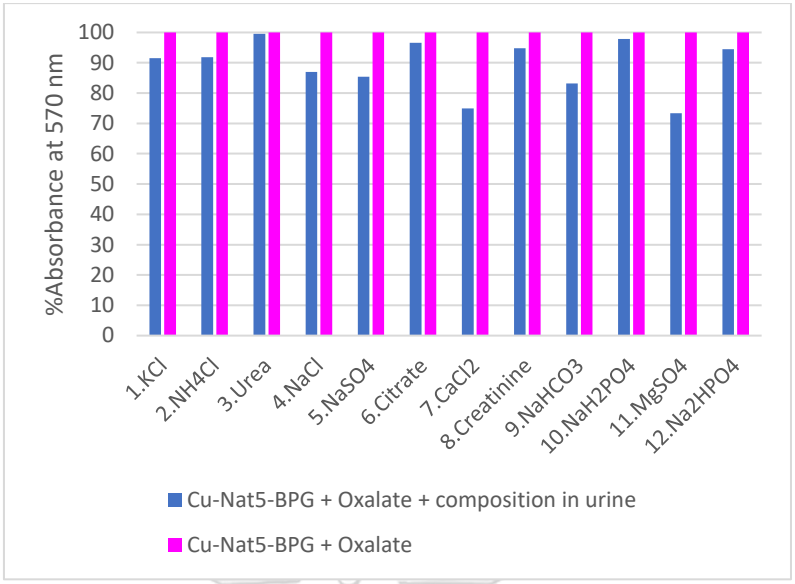


Figure 27. Sensing of oxalate by Cu-Nat5-BPG in the presence of competitive urinary substances;

1. KCl (40 mM), 2. NH4Cl (40 mM), 3. Urea (200 mM), 4. NaCl (200 mM), 5. NaSO4 (10 mM), 6. Citrate (10 mM), 7. CaCl2 (10 mM), 8. Creatinine (10 mM), 9. NaHCO3 (10 mM), 10. NaH2PO4 (10 mM), 11. MgSO4 (10 mM), 12. Na2HPO4 (1.25 mM)

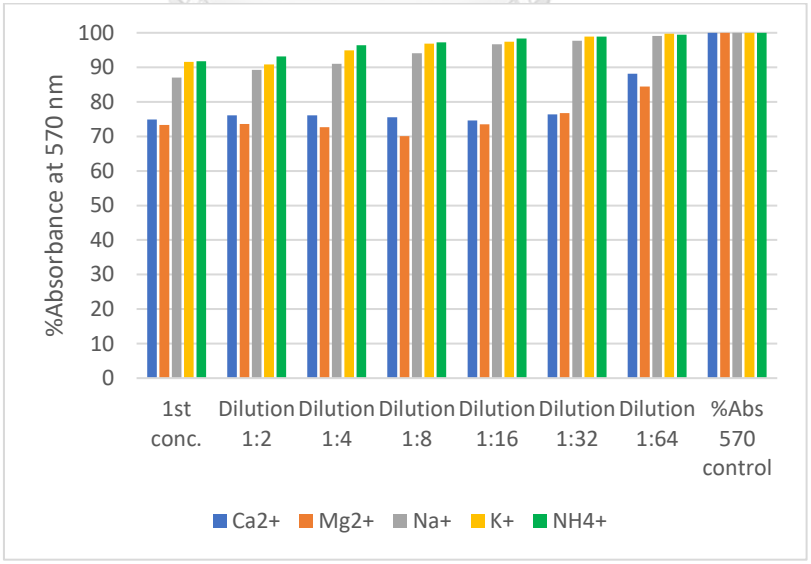


Figure 28. Sensing of oxalate by Cu-Nat5-BPG in the presence of potential urinary interferences; Ca^{2+}, Mg^{2+}, Na^{+} and K^{+} and NH_4^{+} with 6 dilutions from previous experiment.

Part2: Protein expression

2.1 Cloning of the barley-OxOx-PUC57 in the *E.coli* strain DH5α

After, we received the synthesized vector barley-OxOx-PUC57 (4 µg), we propagated them, and kept them as a stock in glycerol (glycerol stock). The barley-OxOx-PUC57 was subsequently cloned into the *E. coli* strain DH5α. The clones were successfully selected on the low salt LB agar plate containing 100 µg/mL ampicillin (Fig. 29).

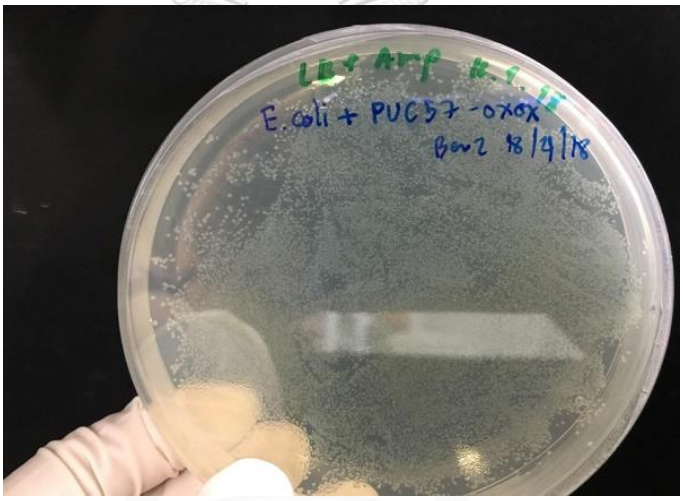


Figure 29. Ampicillin-resistant colonies of transformed E. coli strain DH5α with barley-OxOx-PUC57 vector

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2.2 Isolation of the target DNA (barley-OxOx) from cultured bacterial cells

We used to Presto™ Mini Plasmid Kit to extract and purify the plasmid barley-OxOx-PUC57 from the cultured transformed *E. coli* strain DH5α. The purified plasmid DNA was quantified based on absorption at A260nm/A280nm by NanoDrop™ 2000/2000c spectrophotometers. Barley-OxOx-PUC57 plasmids were double digested by *Xho I* and *Not I* restriction enzymes, and the target barley-OxOx sequence was separated by agarose gel electrophoresis (Fig. 30). The target DNA (634 bp) in the agarose gel was extracted and purified using the QIAquick gel extraction kit.

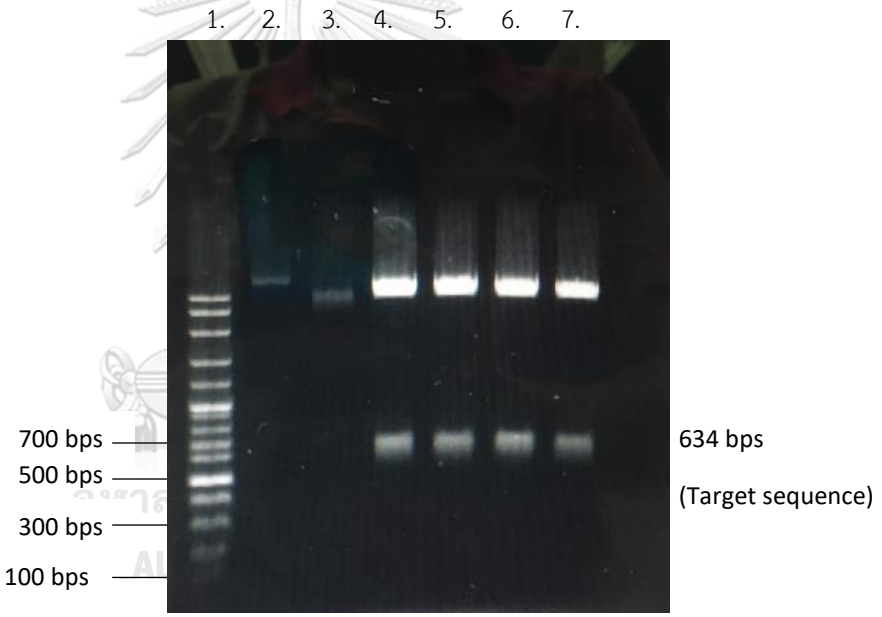

Figure 30. Double digestion of barley-OxOx-PUC57 and separation of digested fragments by agarose gel electrophoresis.

Lane 1: DNA marker, Lane 2: Uncut bayley-OxOx-PUC57, Lanes 3.-7: Double digestion of barley-OxOx-PUC57.

2.3 Propagation and isolation of the expression vector pPICZαA from cultured transformed bacterial cells with Derp8-pPICZαA

The glycerol stock of transformed *E. coli* strain TOP10 containing Derp8-pPICZαA vector was inoculated in LB media with the 100 µg/mL zeocin. We used the same kit as described above to extract and purify the plasmid Derp8-pPICZαA from the cultured transformed *E. coli* strain TOP10. The purified plasmid DNA was quantified using NanoDrop™ 2000/2000c spectrophotometers. The Derp8-pPICZαA plasmids were double digested by the restriction enzymes (*Xho I* and *Not I*), and the DNA fragments of pPICZαA plasmid were separated by agarose gel electrophoresis in the same way as described above. The target pPICZαA plasmid (3000 bp) was extracted and purified using the QIAquick gel extraction kit (Fig. 31).

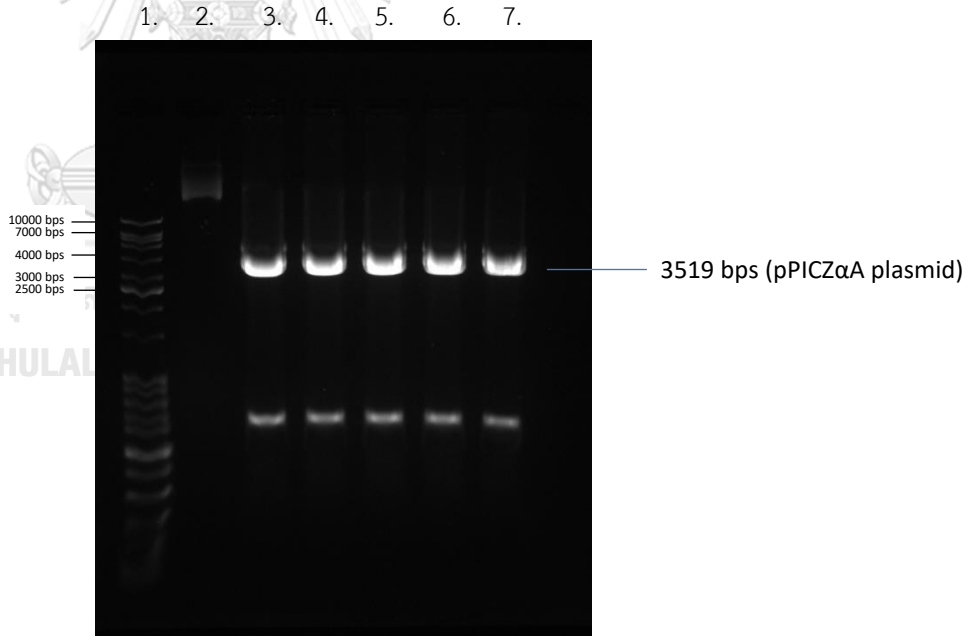

Figure 31. Agarose gel electrophoresis of double digested Derp8-pPICZαA plasmids. Lane 1: DNA marker, Lane 2: Uncut Derp8-pPICZαA, Lanes 3.-7: Double digested Derp8-pPICZαA

2.4 DNA ligation and transformation into *E.coli*

In this study, varied ratios of insert DNA-to-vector (1:1, 3:1, 5:1 and 7:1) were used for ligation. Two 2 strains of *E. coli* were used for the transformation barley-OxOx-pPICZαA vector, DH5α and TOP10 strains. The result showed that both the ratio of DNA insertion-to-vector and strains of *E. coli* influenced the efficacy of vector transformation. Ligation of barley-OxOx and pPICZαA vector at the ratio of 5:1 and 7:1 transformed into the *E. coli* strain TOP10 were selected because they showed the colony growth on LB (low salt) agar plate containing 100 µg/mL zeocin (Table 4, Fig. 32).

Ratio Insert DNA : Vector Stain of E.coli	1:1	3:1	5:1	7:1
DH5α	No growth	Little growth	Little growth	Little growth
TOP10	N/A	N/A	Colony growth	Colony growth

Table 4. The result of varied conditions for ligation and transformation

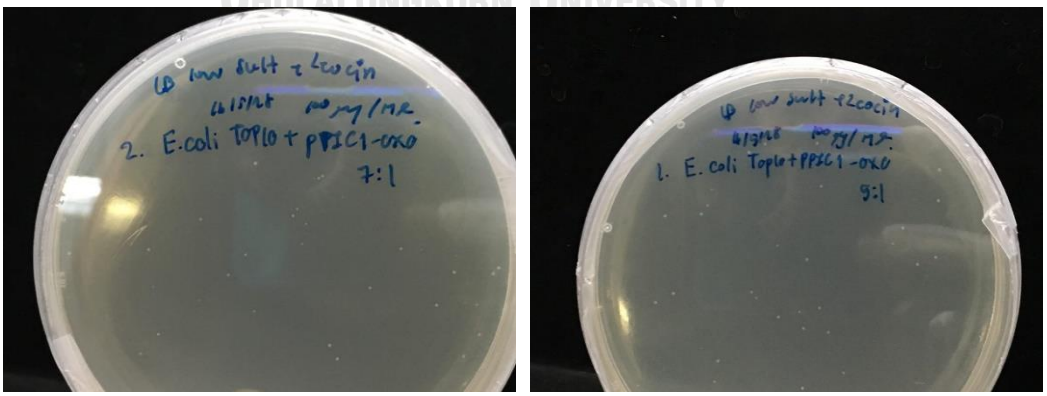

Figure 32. The zeocin-resistant colonies from the ligation of barley-OxOx and pPICZαA vector at the ratio of insert DNA: vector 5:1 (left) and 7:1 (right) into *E.coli* strain TOP10

2.5 Linearization of barley-OxOx-pPICZαA for *P. pastoris* transformation

The expression vector barley-OxOx-pPICZαA was propagated in cultured transformed bacterial cells *E. coli* strain TOP10 on LB (low salt) medium containing 100 μg/mL zeocin. Then the barley-OxOx-pPICZαA was extracted and purified using Presto™ Mini Plasmid kit. The vector was linearized by the restriction enzyme *Sac I*. The result of the vector linearization before transforming into *P. pastoris* strain X-33 (wild-type strain) is shown in Figure 33.

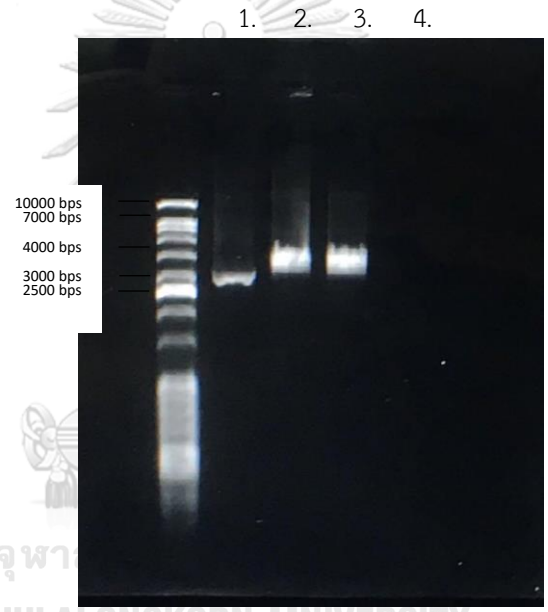

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Figure 33. Linearization of barley-OxOx-pPICZαA shown by agarose gel electrophoresis.

Lane 1: DNA marker, Lane 2: circular barley-OxOx-pPICZαA, Lanes 3-4: Linearizied barley-OxOx-pPICZαA plasmids.

2.6 Transformation of the linearized barley-OxOx- pPICZαA into the *P. pastoris* strain X-33 by electroporation

Linearized barley-OxOx- pPICZαA was transformed into *P. pastoris* strain X-33 by electroporation. The clones were successfully selected on YPD agar plate containing 100 µg/mL zeocin. Then, 26 colonies were re-streaked on YPD agar plate containing 1 mg/ml zeocin for screening multicopy chromosomal integrant and high-level expression (Fig. 34).

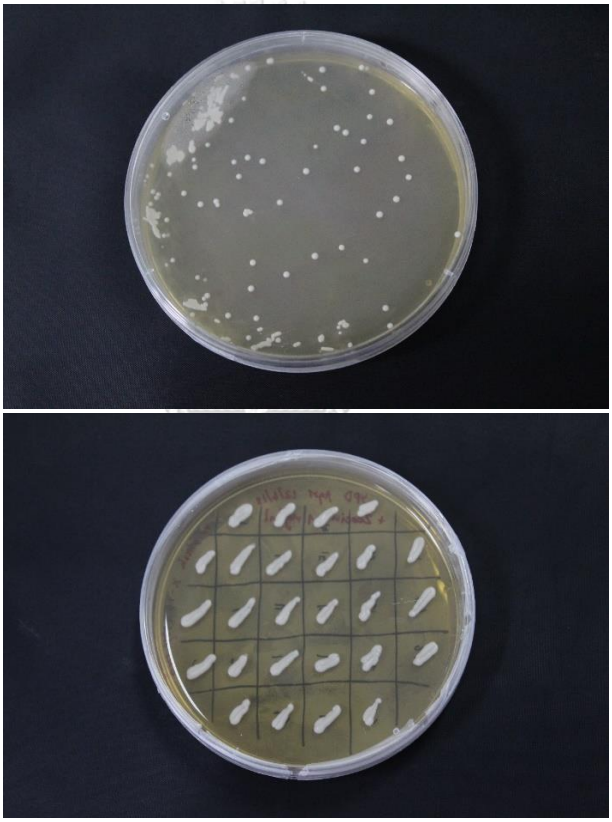

Figure 34. Zeocin-resistant colonies of the strain X-33 transformed with the linearized barley-OxOx- pPICZαA vector
and re-streaked colonies on YPD agar plate containing 1 mg/ml zeocin (lower).

2.7 Barley-OxOx expression assay

Several zeocin-resistant colonies were cultured under shaking condition at 30°C, incubated overnight (14-16 h) in the YPD medium. The cultured cells were centrifuged and transferred into the BMGY medium and cultured again under the same culture condition. When the cells reached a final concentration of OD_{600} nm = 5-10, the cells were centrifuged to remove any trace of BMGY and transferred into BMMY medium at a final concentration of OD_{600} nm = 1 containing 0.5% methanol to induce barley-OxOx expression. Every 24 h, the culture medium was collected, and methanol was re-added to maintain the inducer concentration. The SDS-PAGE showed different patterns of protein expression on SDS-PAGE (each SDS-PAGE came from a different clone, Fig.35 – Fig.39). Theoretically, the monomer molecular mass of oxalate oxidase protein is 26 kDa. Some protein bands inP5, P6 and P9 clones showed the size of about 26 kDa. These bands were selected and excised for the mass spectrometry analysis to confirm whether they are the oxalate oxidase or not. The excised gel contained bands between 26 kDa and 34 kDa in the P5 clone (Fig.35 SDS-PAGE clone P5), at 34 kDa in the P6 clone (Fig36. SDS-PAGE clone P6), and the bands between 34 kDa and 43 kDa in the P9 clone (Fig39. SDS-PAGE clone P9). For P7 and P8 clones, there were no expected bands observed, and therefore, they were not selected for further mass spectrometry analysis (Fig.37-Fig.38 SDS-PAGE clone P7 and P8).

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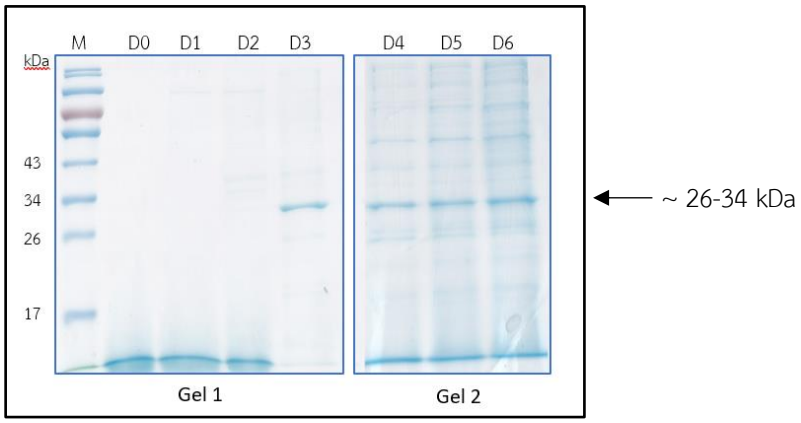

Figure 35. Protein expression of clone P5 in P. pastoris strain X-33 (Day0-Day6).

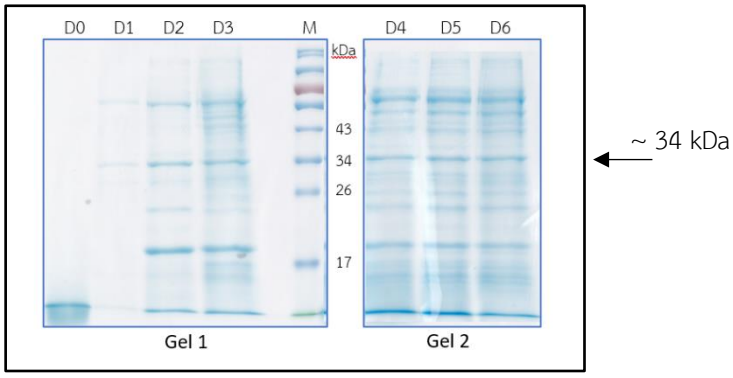

Figure 36. Protein expression of clone P6 in P. pastoris strain X-33 (Day0-Day6).

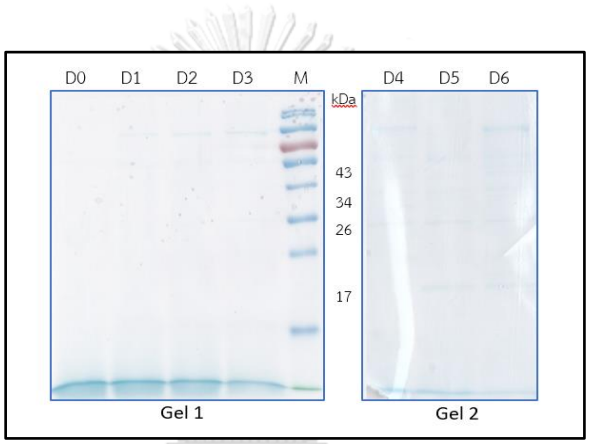

Figure 37. Protein expression of clone P7 in P. pastoris strain X-33 (Day0-Day6).

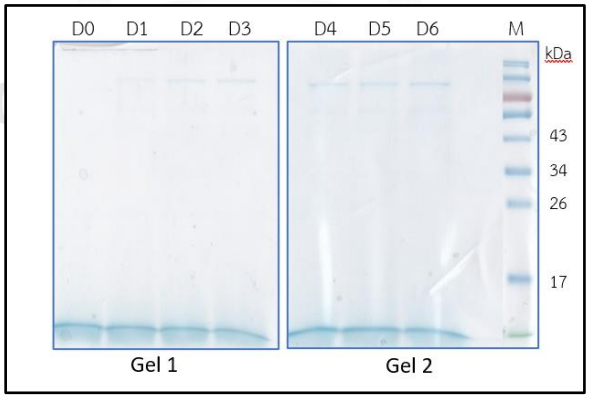

Figure 38. Protein expression of clone P8 in P. pastoris strain X-33 (Day0-Day6).

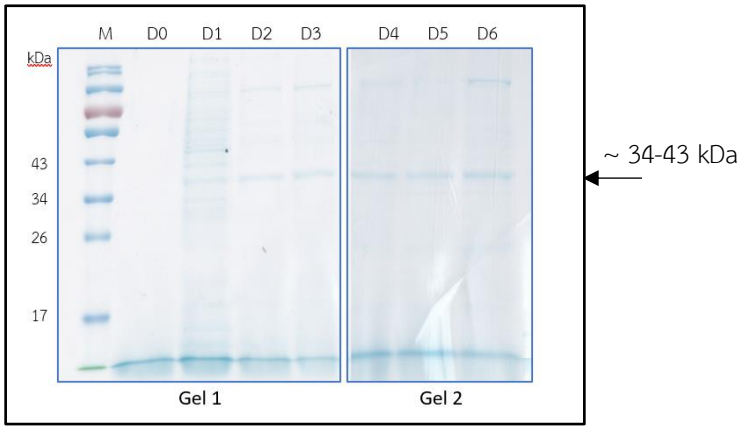

Figure 39. Protein expression of clone P9 in P. pastoris strain X-33 (Day0-Day6).

2.8 Barley oxalate oxidase identification by mass spectrometry analysis

The extracted proteins were digested by trypsin and subjected to mass spectrometry analysis. The enzyme trypsin is used to digest proteins into short chains of peptides at the location of either K (lysine) or R (arginine), except when it is followed by P (proline). The amino acid sequences of these tryptic peptides were determined by mass spectrometry. The result from mass spectrometry showed four tryptic peptides in P5, one tryptic peptide in P6, and two tryptic peptides in P9 samples. These peptides were matched with the *oxalate oxidase 1* (OXO1_HORVU, P45850) and oxalate oxidase 2 (OXO2_HORVU, P45851) (Table 5). According to the database, OXO1_HORVU and OXO2_HORVU are oxalate oxidase proteins of Hordeum vulgare or barley as shown below.

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Protein Accessions P45850 = OXO1_HORVU

- Recommended name: Oxalate oxidase 1
- Alternative name: Germin
- Organism: Hordeum vulgare (Barley)

Protein Accessions P45851 = OXO2_HORVU

- Recommended name: Oxalate oxidase 2
- Alternative name: Germin
- Organism: Hordeum vulgare (Barley)

Expected protein translation of GenBank L15737 matched with result from mass spectrometry analysis is shown as follows:

"SDPDPLQDFCVADLDGK(2)AVSVNGHTCKPMSEAGDDFLFSSKLTKAGNTSTPNGSAVTE LDVAEWPGTNTLGVSMNRVDFAPGGTNPPHIHPR(5)ATEIGMVMKGELLVGILGSLDSGNK (3)LYSRVVRAGETFVIPR(1)GLMHFQFNVGKTEAYMVVSFNSQNPGIVFVPLTLFGSDPPIPT PVLTKALRVEAGVVELLK(4)SKFAGGS"

Table 5. The result from mass spectrometry

Confidence	PSM Ambiguity	Annotated Sequence (sequence number)	Protein Groups	Proteins	Master Protein Accessions	Protein Accessions
Sample name: P5						
High	Unambiguous	AGETFVIPR (1)	1	1	P45850	P45850
High	Unambiguous	SDPDPLQDFCVADLDGK (2)	1	1	P45850	P45850
High	Unambiguous	GELLVGILGSLDSGNK (3)	1	2	P45850	P45850; P45851
High	Unambiguous	VEAGVVELLK (4)	1	2	P45850	P45850; P45851
Sample name: P6						
High	Unambiguous	VEAGVVELLK (4)	1	2	P45851	P45850; P45851
Sample name: P9						
High	Unambiguous	VDFAPGGTNPPHIHPR (5)	1	1	P45850	P45850
High	Unambiguous	AGETFVIPR (1)	1	1	P45850	P45850

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2.9 Recombinant barley-oxalate oxidase purification

After the result of mass spectrometry, the P5 clone was cultured for protein expression on the large-scale cultured medium containing 0.5% methanol for inducing recombinant barley-oxalate oxidase (rBarley-OxOx) expression. Every 24 h, the culture medium was collected, and methanol was re-added to maintain the inducer concentration. In first purification step, we used the CM sepharose bead ion-exchange chromatography (GE healthcare, U.S.A) to purify the rBarley-OxOx, the pH of supernatants containing secreted the rBarley-OxOx was adjusted to pH 3. Our results showed that rBarley-OxOx could bind on the CM sepharose beads and eluted under low salt concentrations. The purification efficacy was checked by SDS-PAGE (Fig. 40)

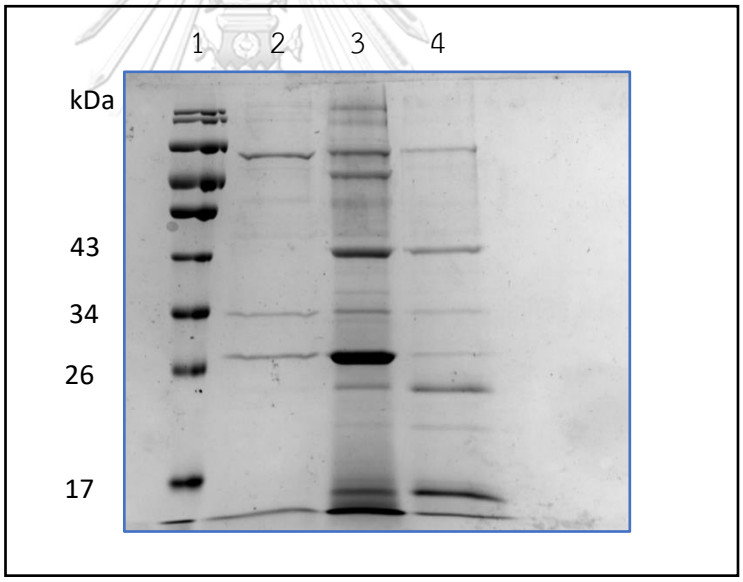

Figure 40. Purification of rBarley-OxOx using CM sepharose bead ion-exchange chromatography

Lane 1: protein marker, Lane 2: starting material, Lane 3: fractions on elution using 100 mM and 200 mM NaCl, and Lane 4: fractions on elution using 300 mM and 500 mM NaCl.

For the second purification step, the fractions containing rBarley-OxOx from the ion exchange chromatography were pooled and concentrated by ultrafiltration using regenerated cellulose membrane with a 10 kDa cut-off. The concentrated protein was applied onto a superdex 75 HR column equilibrated in PBS, pH 7.0. The chromatogram revealed 2 chromatographic peaks which consisted of symmetric peak and non-symmetric peak, suggesting that rBarley-OxOx contained in the non-symmetric peaks (Fig. 41). The SDS-PAGE analysis of fractions 12 and 26-32 confirmed the purity of rBarley-OxOx (Fig. 42).

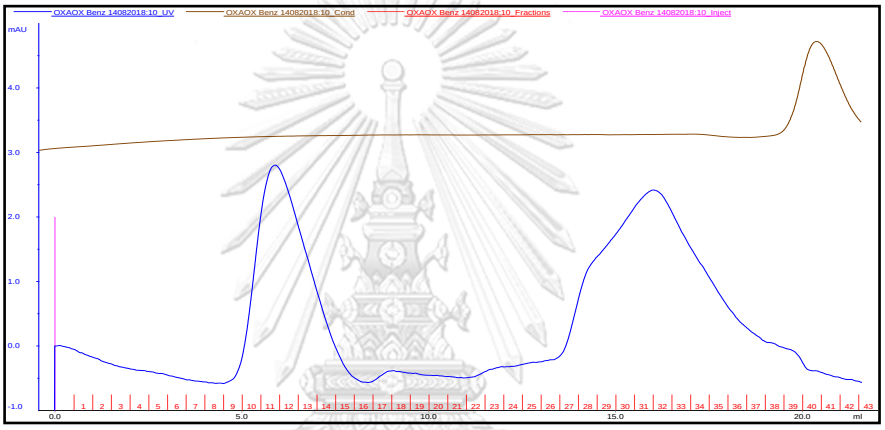

Figure 41. Size exclusion chromatogram of rBarley-OxOx in the purification step.

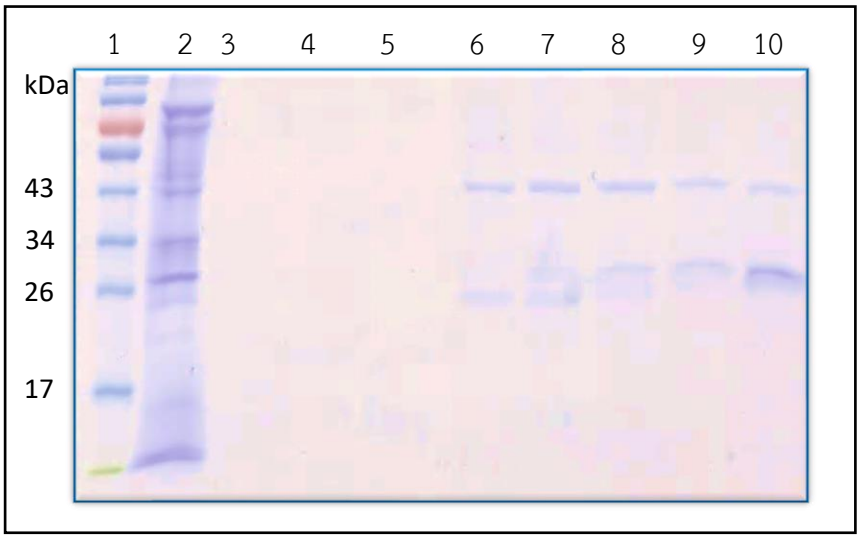

Figure 42. The SDS-PAGE analysis of fractions 12 and 26-32

Lane 1: protein marker, Lane 2: starting material, Lane 3: fraction 12 and Lanes 4-10: fractions 26-32.

The fractions 10-40 of size exclusion chromatography were tested to confirm the OxOx enzyme activity by enzymatic assay for oxalate. The result showed that fraction 29 revealed the peak which could be oxalate oxidase and showed oxalate oxidase activity. Fractions 27, 28, and 30 showed a relatively low signals of oxalate oxidase activity (Fig. 43).

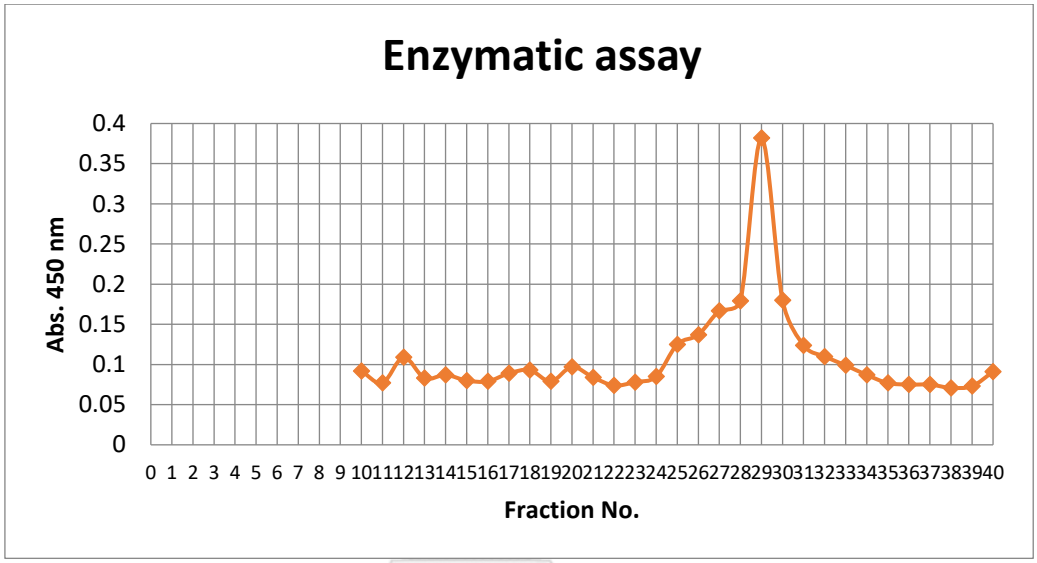

Figure 43. The Graph of the enzymatic assay for oxalate of fractions 10-40.

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Part3: Oxalate measurement by oxalate oxidase

1.1 Characteristic of clinical sample 24hr urine

In this study, a total of 191 24-h urine samples were obtained from 122 non-stone subjects and 69 kidney stone (KS) patients. Types of 69 kidney stone samples were identified by Fourier transform infrared spectroscopy (FTIR), and it revealed that 53 samples (77%) were CaOx stones. The BMI (24.83 ± 3.77 vs. 23.82 ± 4.07 kg/m2) and 24-h urine volume (1327 ± 641.3 vs. 1502 ± 512.1 mL) of the non-stone subjects and stone patients were not significantly different (Table 6). In contrast, age (50.68 ± 9.429 vs. 56.55 ± 10.89 years) and iCOCI (0.4848 ± 0.5766 vs. 2.02 ± 1.662 COM equiv. g/day) in the stone patients were significantly higher than that in the non-stone subjects. Urine creatinine (2.291 ± 2.051 vs. 1.693 ± 1.592 g/day) in stone patients was significantly lower than the non-stone subjects (p value < 0.05) (Table 6).

Table 6. The characteristics of 24-h urine specimens used in the study.

Sample Group	No. of sample	Sex (M:F)	Age (years)	BMI (kg/m2)	Urine volume (L)	Urine creatinine (g/day)	iCOCI (COM (g/day))
Non-Stone sample	n=122	38:84	50.68 ± 9.429	24.83 ± 3.77	1327 ± 641.3	2.291 ± 2.051	0.4848 ± 0.5766
Stone-Patient sample	n=69	47:22	56.55 ± 10.89	23.82 ± 4.07	1502 ± 512.1	1.693 ± 1.592	2.02 ± 1.662
p value	-	-	0.0001**	0.0909	0.0527	0.038*	<0.0001***

- Stone types by FTIR (n=69)
  - Calcium oxalate (CaOx-KS) n = 53 (76.81%)
  - Calcium phosphate (CaP-KS) n = 4 (5.80%)
  - Uric acid (Uric-KS) n = 12 (17.39%)

1.2 The results of oxalate level in 24-hour urine samples by oxalate oxidase method

3.2.1 Urinary oxalate in non-stone forming control group (NS) and kidney stone group (KS)

Comparison of urinary oxalate excretion between the NS group (n = 122) and KS group (n = 69) found that the KS group was urinary oxalate was significantly higher than in the NS group (11.50 ± 1.421 vs. 7.261 ± 0.703 mg/day) (*p=0.0032*) (Figure 44).

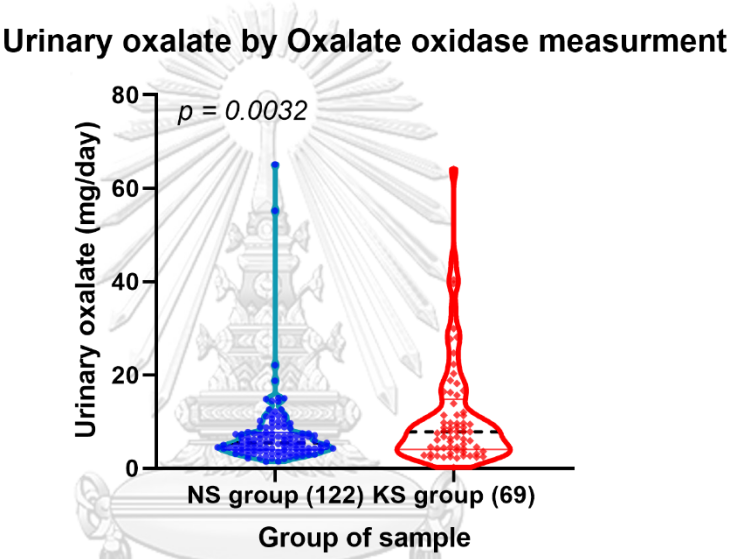

Figure 44. Level of urinary oxalate excretion in the NS group was significantly lower than the KS group.

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3.2.2 Urinary oxalate in NS group and KS group regarding to type of stones

Measurement of urinary oxalate compared among the NS group (n = 122), CaOx-KS group (n = 53), CaP-KS group (n = 4) and Uric-KS group (n = 12) found that the urinary oxalate in CaOx-KS group was significantly higher than the NS group (12.15 ± 1.765 VS. 7.261 ± 0.703 mg/day) (*p=0.0022*). By contrast, the urinary oxalate in NS group, CaP-KS group (10.71 ± 5.767 mg/day) and Uric-KS group (8.914 ± 1.886 mg/day) were not significantly different (Fig. 45).

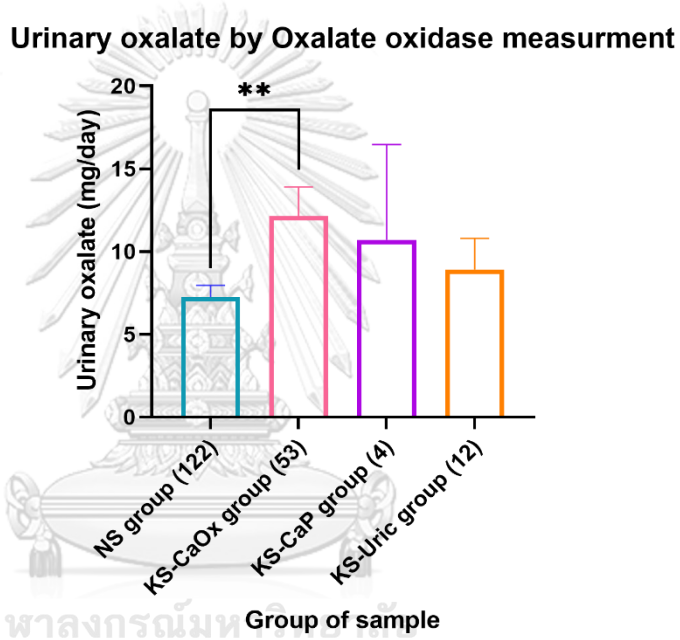

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Figure 45. Comparison of urinary oxalate the NS group and kidney stone group according to the type of stones.

1.3 Urinary oxalate measurement compared between OxOx method and HPLC technique

3.3.1 Oxalate measurement in water (standard oxalate)

In this study, we measured the urinary oxalate by HPLC using Rezex™ ROA-Organic Acid H+ LC column, sized 300 x 7.8 mm. Oxalate peak found at approximately 8.73 minute, however the time may vary slightly depending on the composition of the solution. (Fig. 46)

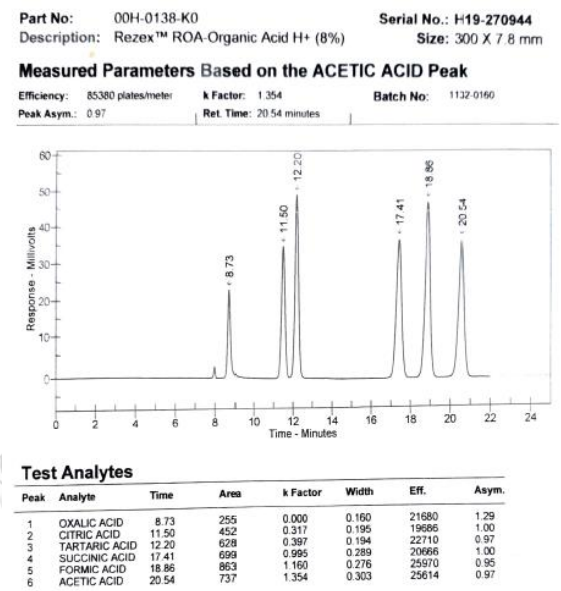

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Figure 46. The oxalate standard peak of Rezex™ ROA-Organic Acid H+, LC Column 300 x 7.8 mm. by HPLC

Oxalic acid dissolved in water was measured by HPLC and OxOx method at various concentrations as follows: 1, 0.5, 0.25, 0.125, 0.0625, 0.0312 and 0.0156 mM. It was found that the peak height and the peak area of oxalate (at approximately 8.73 minute) increased as the oxalate concentration increased (Fig. 47). Likewise, the standard curve of oxalate measured by OxOx method also showed the perfect linearity, as the absorbance (650 nm) increased proportionately with the oxalate concentrations (Fig. 48).

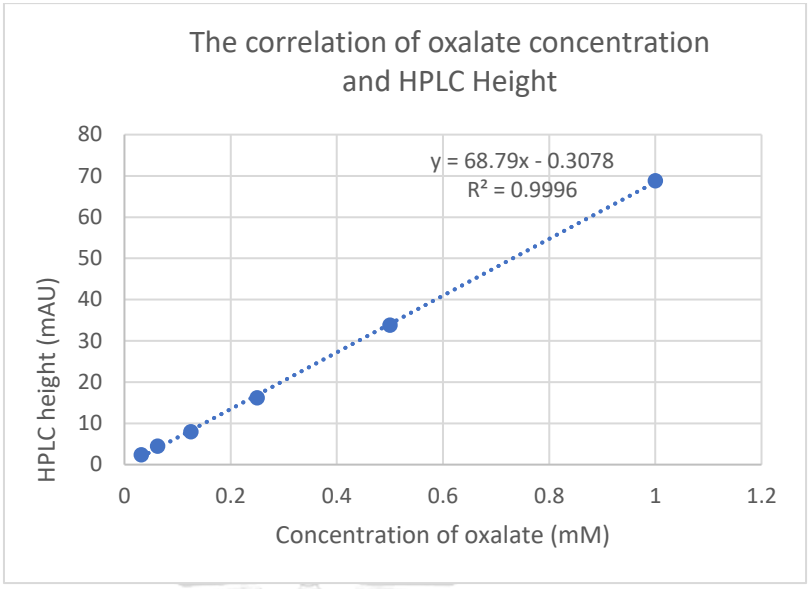

Figure 47. The correlation of oxalate concentration and HPLC peak height

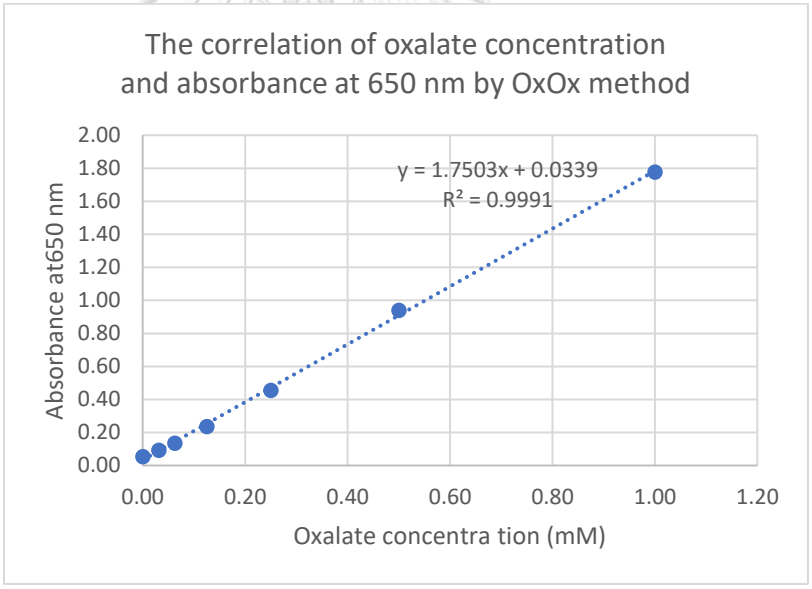

Figure 48. The correlation of oxalate concentration and absorbance at 650 nm measured by OxOx method

3.3.2 Oxalate levels in clinical samples compared between measurements by OxOx and HPLC techniques

To evaluate whether the urinary oxalate measurement by our newly established OxOx enzymatic method was accurate compared with the HPLC method, urinary oxalate concentrations were measured in 191 24-h urine samples both by OxOx and HPLC methods. The results showed that the urinary oxalate levels in all urine samples measured by OxOx method were significantly lower than those measured by the HPLC techniques (Table 7, and Fig.50). However, the levels of urinary oxalate of both methods were significantly correlated (R^2 = 0.51, R = 0.7143) (Fig. 50). In NS-group, urinary oxalate measured by HPLC was significantly higher than the OxOx method (Table 7, and Fig.50). The correlation of the urinary oxalate in NS-group between HPLC and OxOx method statistically correlated, but with a weaker correlation (R^2 = 0.26, R = 0.5099) (Fig. 51). Perhaps, it was due to the level of oxalate in normal urine was usually low, and it might be below the limit of detection in some cases.

In contrast, the urinary oxalate concentration in KS-group measured by HPLC and OxOx methods were not significantly different (Table 7, and Fig.50). Furthermore, the levels of urinary oxalate determined by these two methods were highly correlated (R^2 = 0.88, R = 0.9387) (Fig. 52).

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Table 7. The detail of results of urinary oxalate divided by group and method

Group of Samples	NS-group		KS-Group		All	
Method	By OxOx	By HPLC	By OxOx	By HPLC	By OxOx	By HPLC
Number of values (n)	122	122	69	69	191	191
Minimum (mg/day)	1.44	0.846	0.207	0.106	0.207	0.106
Maximum (mg/day)	65.09	60.26	64.09	62.17	65.09	62.17
Mean	7.261	10.69	11.5	11.51	8.793	10.99
Std. Deviation	7.77	9.146	11.8	11.04	9.616	9.852
Std. Error of Mean	0.7035	0.828	1.421	1.329	0.6958	0.7129

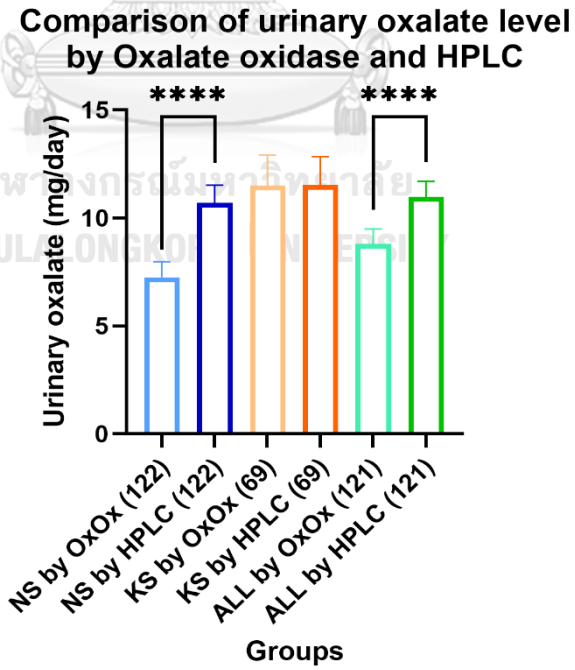

Figure 49. Comparison of urinary oxalate in mg/day between groups and methods

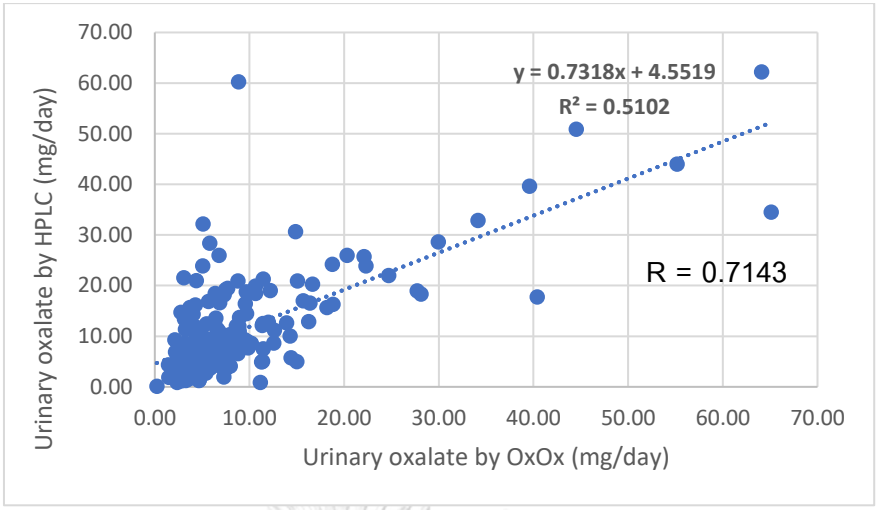

Figure 50. The correlation of urinary oxalate measured by OxOx and HPLC in all urine samples

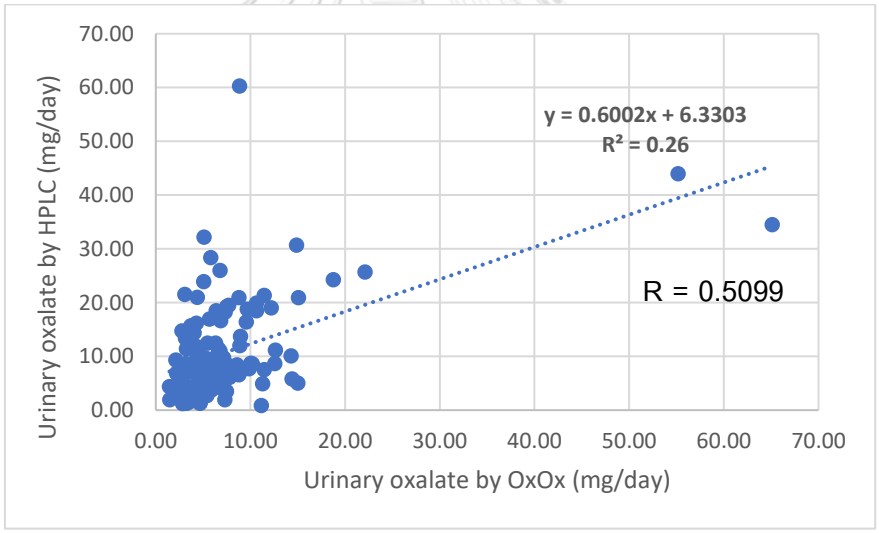

Figure 51. The correlation of urinary oxalate measured by OxOx and HPLC in NS group

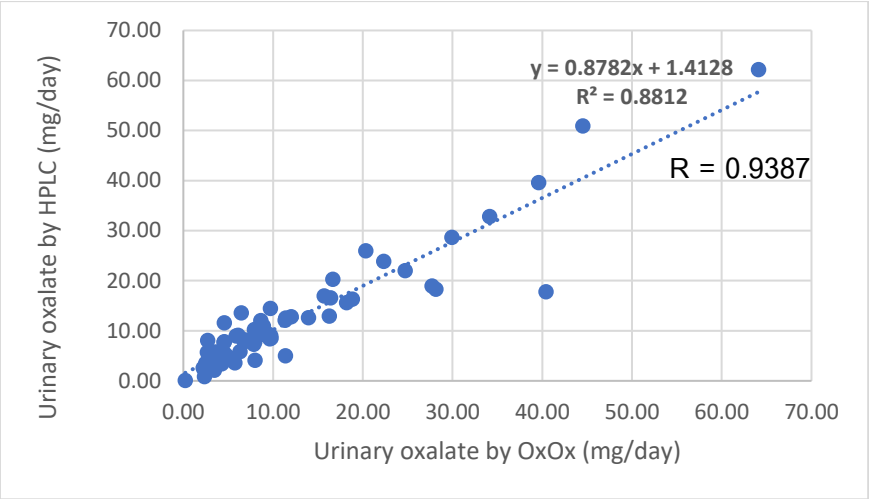

Figure 52. The correlation of urinary oxalate measured by OxOx and HPLC in KS group

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Chapter 5

Discussion and Conclusion

In this study, we focused on development of the new urinary oxalate measurement. Urinary oxalate is an indicator of CaOx kidney stone formation, which is the most common type of kidney stones (60-80%). We tried first to develop the new non-enzymatic methods for oxalate detection using the ensemble of Cu-Nat5-BPG. It turned out that measurement of oxalate by Cu-Nat5-BPG ensemble was interfered by many urinary constituents, especially cations (Ca^{2+} and Mg^{2+}). Therefore, we concluded in this study that the Cu-Nat5-BPG ensemble was not suitable for detecting oxalate in urine samples primarily due to the interfering ions in urine samples. We therefore tried to produce our own OxOx enzyme for establishing our own in-house OxOx method for oxalate measurement. We successfully cloned and expressed recombinant barley OxOx in yeast cells, but the expression yield was very low. We concluded that although recombinant OxOx could be laboratory produced, but it was not feasible for upscaling to be commercially useful. Finally, we purchased the commercially available OxOx to be used for establishing our own in-house OxOx method for urinary oxalate measurement. We showed that urinary oxalate measurement by our in-house OxOx method was as accurate as HPLC technique, and it was clinically useful.

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Our first goal was that to develop the POCT test based on the Cu-Nat5-BPG ensemble. The Cu-Nat5-BPG was perfectly demonstrated to be a biosensor for oxalate in a 80% acetonitrile solution buffer with 10 mM HEPES at pH 7.0. After addition of oxalate into the solution of the Cu-Nat5-BPG ensemble, it turned the color from blue of the Cu-Nat5-BPG to pink of free BPG. Competition between the dye indicator and the specific substance in the receptor or ligand is known as an indicator displacement assay (IDA).

We studied the specificity of oxalate and ensemble against various anions. Cyanide (5mM), PPi (25mM), and creatinine (17.68 mM) showed a significant interfering effect. To make the test more specific to oxalate, these urinary substances must be removed. It must be noted that creatinine is one of the most important components in urine, and it is hard to remove. Moreover, we also demonstrated that common urinary cations, specifically calcium (10 mM) and magnesium (10 mM) significantly interfered the binding of oxalate to the Cu-Nat5-BPG. Additionally, even though the urine sample was diluted to 1:32 or 1:64 the amount of calcium and magnesium in the urine still interferes with oxalate binding to the ensemble. The amount of oxalate in normal urine is relatively low, usually less than 0.46 mmol/1.5 L or 0.3 mM. Therefore, if the urine must be diluted to minimize interfering effects of calcium and magnesium, the oxalate will be undetectable due to the limit of detection of the test (oxalate is too low to be able to measure). The detection limit of the Cu-Nat5-BPG method was 0.02 mM [17].

We further aimed to produce the rBarley-OxOx as a recombinant protein from *Pichia pastoris* [18] to reduce the cost of future purchases of commercially available OxOx. We could express the rBarley-OxOx in *Pichia pastoris*. The purified recombinant protein was confirmed to be the rBarley-OxOx by mass spectrometry. However, the big issues were that the expressed amount of rBarley-OxOx was limited, the yielding amount was not inadequate for further experiment, it was relatively difficult to reproduce it, and it was hardly possible to upscale for commercial utility. We had contacted the corresponding author of the previous report (Prof. Ellen W. Moomaw) [18] on cloning of OxOx, and she said “I need to warn you that just as Dr. Whitaker reported and as I reported the yield is very, very low. Also, as my construct is not His-tagged the purification is laborious to yield approx. 10-20 mg/L.”. This underlined our finding and conclusion that cloning and expressing the recombinant OxOx was possible, but amount of production and cost of purification remained challenging and unsolved. However, the most specific and effective method for measuring oxalate in urine samples

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was the enzymatic method based on OxOx. It is still worthwhile to seek alternative sources of OxOx or the other effective ways to produce recombinant OxOx. The potential sources of OxOx could be cloning and expressing recombinant OxOx in plant cells or purifying the naturally produced OxOx from the OxOx-producing plants such as barley seedlings, mosses, beet stems, and wheat seedings.

The results of urinary oxalate measurements by HPLC and enzymatic method showed well agreement. Both methods showed that urinary oxalate level in non-stone subjects was significantly lower than in kidney stone patients. Based on correlation test, urinary oxalate level measured by OxOx method was correlated well with those measured by HPLC, and the correlation was more pronounced in the kidney stone group. It should be noted that correlation of urinary oxalate levels between both methods was weaker in the NS-group (R^2 = 0.26) than the KS-group (R^2 = 0.88). This could be explained by a relatively low concentrations of oxalate in healthy urine, and in most cases, the oxalate levels were lower than the measurement limit, that would cause a variability and inaccuracy in the measurement.

Finally, we employed the commercial OxOx for establishing the in-house procedure for urinary oxalate measurement. This study demonstrates that our in-house OxOx method could be clinically used to separate the stone patients from healthy individuals, and it could be clinically beneficial for estimating the risk of kidney stone development.

The limitations of this study should be mentioned. Oxalate measurement studied in the urine samples in 191 urine samples only from Maha Sarakham Province, Thailand. The urine samples were collected in 2016 and kept at -80^{o}C. It might be that the urine samples had been kept too long, and it could affect the actual amount of oxalate in urine.

In conclusion, we successfully establish the in-house protocol for urinary oxalate determination using the commercially available OxOx coupled with HRP. The unit cost of our in-house oxalate testing was cheaper than the commercial oxalate test kit. Based on our finding, production of recombinant OxOx by cloning and expressing in *P. pastoris* was not recommended because of very low production yield. Other host system could be the solution for this. Probably, the recombinant OxOx could be cloned and expressed in plant cells to achieve the high production yield. Alternatively, optimization of the types of expression vectors and expressing procedure would be the way to increase the production yield. In addition, OxOx can be obtained from the natural source. Purification of the naturally produced OxOx from plants such as barley seedings [35], banana peels [54], maize roots [38], *Lasia spinosa* (Pak-Nam) would be an alternative. In fact, most of OxOx that are commercially available and OxOx that are used in the published papers are the OxOx purified from plants.

In the future, we have planned to develop and look forward to the successful development of point-of-care testing (POCT) for urinary oxalate detection based on OxOx and HRP. We do hope that this proposing oxalate POCT could be able to empower people to assess the risk for CaOx kidney stone by themselves (or their loved ones) at home without a need to come to the hospital or laboratory.

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APPENDIX

Reagents used in this study

1. Oxalate detection by ligands Cu-Nat5

1.1 50 mM HEPES buffer, pH 7.4 (stock)

- HEPES (MW = 238.30) 11.915 g
- Distilled water (DW) 800 mL

Adjust pH to 7.4 and adjust volume to 1 L with DW

1.2 10 mM HEPES buffer, pH 7.4 in 80% acetonitrile solution

(prepared 80:20 acetonitrile: water)

- 50 mM HEPES buffer, pH 7.4 200 mL
- Acetonitrile 800 mL

2. Oxalate detection by ligands oxalate oxidase

2.1 50 mM succinate buffer, pH 3.8

- Succinic acid (MW = 118.09) 5.904 g
- Distilled water (DW) 800 mL

Adjust pH to 3.8 and adjust volume to 1 L with DW

2.2 Working TMB, 0.1 mg/mL

- Stock TMB, 1 mg/mL, store at -20 ^{o}C 1 mL
- Succinate buffer pH 3.8 9 mL
- Store 4 ^{o}C in dark container

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