

Characteristics and heterologous expressions of oxalate degrading enzymes “oxalate oxidases” and their applications on immobilization, oxalate detection, and medical usage potential

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Oxalate oxidase (OxO) is an enzyme that catalyzes oxalate to hydroperoxide (H₂O₂) and carbon dioxide (CO₂). Besides its role in cell morphogenesis, OxO mainly exists in the cell wall to enhance resistances to diseases and other environmental stresses in plants. While in mammals, oxalate is a dead-end metabolite which causes hyperoxaluria and urolithiasis when in excess. OxOs found in bacteria, fungi, and high plants have a number of potential applications, including the medical diagnosis and prophylactic treatment for hyperoxaluria, urolithiasis, the food safety, and even the paper-making industry. In this review, OxO structure, function, immobilization, enzymatic determination of oxalate contents, and clinical and prophylactic supplementation therapy potential were discussed, and the relative progress of the research was also elucidated.

Keywords: Oxalate oxidase; heterologous expression; oxalate determination; medical application.

Abbreviations: OxO: oxalate oxidase; His: histidine; Cys: cysteine; Glu: glutamic acid; Asp: aspartic acid; *E. coli*: *Escherichia coli*; *P. pastoris*: *Pichia pastoris*; HPLC: high performance liquid chromatography; PVC: plasticized polyvinyl chloride; SIRE: sensors based on injection of the recognition element; SMB: simulated moving bed; PEG: polyethylene glycol.

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Introduction

Oxalate oxidase (OxO, oxalate: oxygen oxidoreductase, EC 1.2.3.4) is an enzyme catalyzing the aerobic oxidation of oxalate into H₂O₂ and CO₂. Since its first discovery in moss by Datta *et al.* [1] in 1955, OxO has been found in bacteria, fungi, and plants, e.g., white rot basidiomycete [2], wheat [3], barley [4], sorghum [5], and a number of other higher

plants including *Amaranthus spinosus* [6], spinach beet [7], banana [8], and azalea [9]. However, most of the knowledge gained from the researches on OxOs was from barley and wheat. For example, the active sites of those OxOs were identified convincingly by chemical modifications [10]. Another notable progress is that the crystal structure of barley OxO was determined [11]. The crystallographic data along with the biochemical evidence support

that OxO is a thermal stable homohexameric glycoprotein, and that each subunit contains one manganese ion and one glycan with *N*-glycopeptide linkage to protein through an Asp residue.

Oxalate is a simplest dicarboxylic acid in nature, and widely existed in plant and animal cells. In some *Gramineae* cereal plants like wheat, barley, and sorghum, oxalate can be decomposed by OxO to produce H₂O₂, which can, at moderate concentrations, regulate developmental signals, participate in cell wall cross-linking, and ameliorate environmental stresses. A recent study reveals that OxO modifies ferulate metabolism in cell walls for cell wall cross-linking by promoting peroxidase action for supply of hydrogen peroxide [12]. However, mammals including humans lack endogenous OxO, oxalate is therefore a dead-end substance which cannot be metabolized further. This material with no nutritional value circulates in blood in the anion form, and may accumulate in forms of oxalate crystals in the target tissues, causing serious chronic diseases such as kidney calculus and arthritis in humans [5, 13, 14].

The dietary oxalate is the main contributor responsible for oxalate accumulation in humans [15]. For those who are prone to kidney stone, food with low oxalate contents is strongly recommended. The dietary oxalate was shown in experimental models to be highly correlated with the occurrence rate of oxalate calcium crystalluria and nephrolithiasis [16]. Unfortunately, some of the frequently consumed foods in our daily life are indeed rich in oxalate, such as spinach, bamboo shoots, tea, strawberry, cocoa, chocolate, and some other tropical fruits [17, 18, 19]. Another contributor for oxalate accumulation is the endogenous synthesis pathway, in which oxalate can be synthesized from ascorbate, glyoxylate, or other precursors generated in the protein metabolism. Furthermore, high glucose ingestion can increase the oxalate concentration, even surgical operations may

cause the increment of urinary oxalate production [20].

Owing to the unique property of decomposing oxalate, OxO can be used as a tool enzyme for analysis of oxalate contents and a potential biomedicine. The measurement of oxalate in biological fluid or food is important because the dicarboxylic anion is the main factor for forming kidney stones, or causing other correlative diseases. Human urinary oxalate concentration is an often used indicator to evaluate the potential risk of nephrolithiasis. Some analytical methodologies, such as gas chromatography, high-performance liquid chromatography, and capillary electrophoresis, have been employed for the analysis of oxalate for many years. But those methods have problems of oxalate losses during pretreatment processes [21, 22, 23]. Although enzymatic techniques use less complicated procedures and less expensive equipments and are more popular recently in clinical laboratories, the sensitivity of detection is a big concern. With the emerging biosensors made from OxOs, the sensitivity and specificity issues will be tackled eventually. As a potential source for medical care and supplementation therapy for oxalate-related diseases in humans, many attempts have been made in decades and some exciting breakthroughs were brought out. To promote appreciation of OxO, this review covers OxO characterizations, OxO immobilization, oxalate determination techniques, the potential enzyme therapy for oxalic metabolism disorders, and the forth-looking of the relative researches.

OxO characteristics

Research on the characterization of OxO, including information of structure, active site, and kinetics, is the foundation of its application. Despite being identified and characterized from various *Gramineae* crops, germins attracted a great interest only when this substance from barley and wheat were found to possess OxO activities with unusual stability.

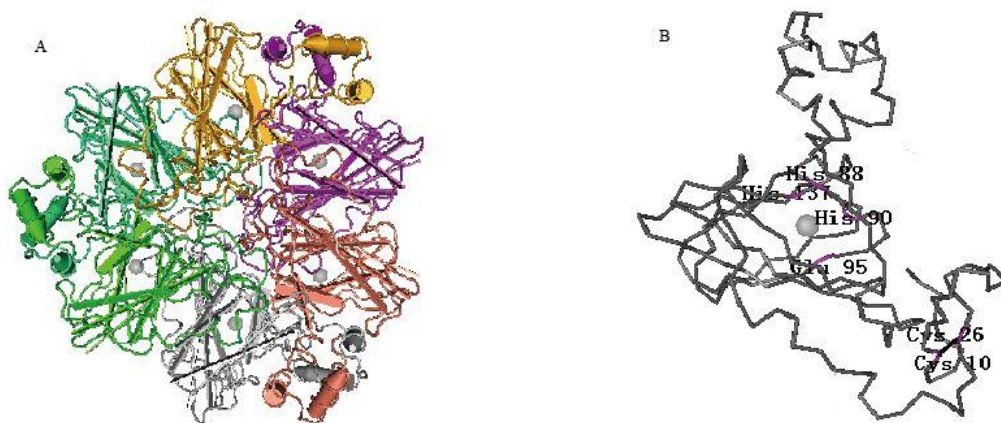


Figure 1. The 3-D structure of barley OxO (NCBI MMDB ID: 36482). **A**, the OxO is homohexameric with trimer of dimer which is clasped by α -helicals (arrows), and with one manganese ion (sphere) in each subunit. **B**, view of OxO subunit, Cys10 and Cys26 form an internal disulfide bond (black line). Glu95, and one manganese ion bonded with three His (His88, His90, and His137) form OxO active site. The figure was drawn by CN3D ver. 4.3.1 (NCBI, USA).

OxO and germins

The first germin found in barley during seeds germination did not match any of the known proteins in databases at that time. In 1993, germins from barley and wheat were first identified to be OxOs [3]. Subsequently, a number of other germins from a subset of *Gramineae* cereals such as oat, ryegrass, and maize were reported to be OxOs. Germins identified so far have 90%-95% identity at the protein sequence level and all exhibit OxO activities. All germins are thermostable oligomeric proteins (e.g., ψ G, an isoform of germins from wheat, almost keeps its full activity at 100°C boiling water for 30 min [5]). Moreover, germins are refractory to dissociation in sodium dodecyl sulfonate (SDS) and resistant to hydrolysis by all known digestive enzymes.

OxO structure and properties

Only a few OxOs have been characterized in relatively more detail at the structural and biochemical levels. Barley OxO was successfully crystallized and its 3-D structure was derived from X-ray at 1.7 Å resolution [11, 24]. This OxO is homohexameric and organized as a trimer of dimers. Each subunit links one glycan and its molecular weight is 22 kDa [25] (Figure 1A). In each subunit, there are two Cys residues to

form an internal disulphide bridge, and the active site is involved with one Glu residue and one manganese ion interacting with three His ligands (Figure 1B). Three residues (two Glu residues and one Asp residue), which can be negative charged at neutral pH, are located at the gate of channel, therefore retard oxalate anions to get close to the active site. It explains the reason that OxOs work at low optimal pH from 3.2 to 5.0 [26, 27] when the three residues are less likely carrying negative charges. Another well studied OxO was from wheat. Results from the chemical modifications showed that Cys and carboxylate residues are essential for its activities as well, similar to those in the barley OxO [10].

Sufficient soluble germins were purified from barley or wheat during germination or maturity periods to gain OxO structure information. However, the purification process is highly costly, labor intensive and with quite low yield, hampering OxOs as a routine tool in the urinary and plasma oxalate detections. Interestingly, an insoluble form of OxO found abundantly in seedlings or brans of cereals might be an alternative solution because its OxO is bounded to cell wall and could be a rich source for a large quantity. Some effort is on the way to explore this rich source. Presently, soluble OxOs from

plants are still the major source for applications such as enzymatic analysis of urinary and plasma oxalate.

The key parameters of the OxOs are K_m , V_{max} , and sensitivity to anions such as Cl^- and NO_3^- in urine and plasma. The ideal OxO for application should have low K_m , high V_{max} for oxalate, and not inhibited by anions in the biological fluids. K_m of wheat OxO for oxalate is 0.21 mM, and K_m of barley OxO for oxalate is between 0.27 mM and 0.42 mM [10]. However, OxOs from both are sensitive to Cl^- and NO_3^- . Encouragingly, OxO from *Amaranthus* leaves is only slightly inhibited by HCO_3^- , and not inhibited by other common anions, but unfortunately has a high K_m for oxalate (2.16 mM) [6]. To date, the limited success of the enzymatic methods for oxalate analysis has been solely relied on those inadequate OxOs endogenously produced by plants.

Heterologous expression of OxO

Since unsuitability of OxOs from natural sources including wheat, barley, and sorghum is an obstacle for their clinical and industrial applications, it is of great interest to engineer desired properties of OxOs and to produce them in a large quantity and at a lower cost. Two heterologous expression systems have been attempted: prokaryotes (e.g., *Escherichia coli*) and eukaryotes (e.g., *Pichia pastoris*). A list of OxO genes registered in GenBank of NCBI database includes wheat germin *gf-2.8* (Acc. No. M63223), wheat germin *gf-3.8* (Acc. No. M63224), barley OxO mRNA (Acc. No. Y14203 and Acc. No. L15737), raygrass OxO mRNA (Acc. No. AJ492380, AJ492381, AJ504848, AJ291825), and *Ceriporiopsis subvermispota* OxO gene (Acc. No. 54019687, AJ746412). Among them, *gf-2.8* and barley OxO mRNA were most frequently chosen for heterologous expression purpose.

Prokaryotic expression

E. coli is a routine tool for a rapid and economical production of recombinant

proteins. However, exogenous proteins expressed in the system often form inactive inclusion bodies. The main challenge is therefore to obtain proteins in soluble and active forms. The endogenous OxOs are homomultimeric glycoproteins with each monomer containing one manganese ion and one properly formed disulfide bond. The proper glycosylation is the result of coordinated processes in organelles including the endoplasmic reticulum, the Golgi apparatus, and the vacuole of cell [28]. Prokaryotes like *E. coli* lack these organelles for modification such as glycosylation. Although the glycosylation of protein is not strictly required for enzymatic activity, the expressed OxOs in *E. coli* without quaternary structure and proper polysaccharides are not stable [5]. Another challenge is that the reducing environment in prokaryotic cytoplasm hinders the formation of the disulfide bond essential for OxO activity. Cassland *et al.* [29] used OrigamiB (DE3) in order to get active OxOs. This special *E. coli* strain harbors *trxB* and *gor*, a double mutant which disrupts the reducing power of the cytoplasm and therefore allows the disulfide formation in the OxOs. The authors expressed the full length of wheat germin *gf-2.8* (Acc. No. M63223) and barley OxO mRNA (Acc. No. Y14203) in OrigamiB (DE3). They did detect part of the enzymatic activities in supernatants, but found the majority of the expressed proteins appeared to be insoluble. Moreover, there were inhibitory agents in the *E. coli* lysates. Although many attempts were made to overcome these obstacles, no useful OxOs have been successfully made from *E. coli*.

Eukaryotic expression

The eukaryotic expression systems include yeast and high plants. *Pichia pastoris*, a yeast frequently used eukaryotic expression system, has the capacities to perform post-translational modifications including signal peptide processing, proper peptide folding, disulfide bond formation, and glycosylation. This expression system was primarily used for high yield of proteins from vertebrate origin [30]. Recently, it has also been successfully used to

produce active proteins of bacterium and plant origins with high yield [31, 32].

Whittaker and Whittaker [33] artificially synthesized a gene based on the barley OxO protein sequence (Acc. No. 289356) and expressed it in a *P. pastoris* expression system. The protein obtained was a soluble, hexameric glycoprotein with native mass of 140 kDa, and each monomer correctly contained one manganese ion and formed the required disulfide bond. The expressed OxO had similar property and structure to the endogenous barley OxO. Furthermore, the yield was as high as 250 mg OxO from material of 5 L fermentation medium. More recently, Pan *et al.* [34] reported a high-level secretory expression of wheat germin *gf-2.8* in *P. pastoris*, and it could serve as a starting point for large scale OxO production. They found that two different weights of manganese-containing monomers were produced, i.e. 23 kDa and 26 kDa. Interestingly, only the 26 kDa monomers were glycosylated. The purified protein formed from 26 kDa monomers was similar to the wheat OxO in characteristics, and the yield was as high as 1.0 g purified protein from material of 5 L fermentation medium. In addition, its activity can be dramatically increased by agents such as periodate and ascorbate.

Theoretically, transgenic plants could be used to obtain abundant recombinant OxO, and indeed such attempts have been made in the recent decade. OxO genes of wheat or barley were expressed in various host plants such as tobacco [35], soybean [36], sunflower [37], maize [38], potato [39], and peanut [40]. However, these transgenic plants produced recombinant OxOs with enzymatic activities only when plants were exposed to environmental or biotic stresses.

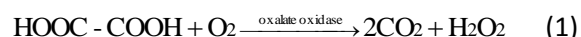
Interestingly, an OxO gene (Acc. No. AJ746412) isolated from a white rot basidiomycete fungus *Ceriporiopsis subvermispora* was successfully expressed in *P. pastoris*. The product of this gene expression had OxO activities. However, it

is not a homologue of OxO, but a homologue of oxalate decarboxylase [41].

OxO immobilization and oxalate detection

The enzymatic method with use of OxO to determine oxalate contents is a fast and convenient way, and it is widely accepted in the field of the medical diagnosis, food safety, and light industries. For example, OxO is employed to avoid the formation of calcium oxalate incrusts in pulps and paper industry, and even it is also used to detect tetrodotoxin [29, 42].

OxO catalyzes the reaction as in the following equation:



The determination of oxalate content by the traditional enzymatic method is based on the coupling hydrogen peroxide to the coloration developed by agents, such as 4-aminoantipyrine, 4-chloro-1-naphthol, and 3-methyl-2-benzothiazolinone hydrazone. Based on this principle, researchers also made use of existing instruments including glucometer to measure oxalate contents by using OxO and peroxidase [43]. The coupling reaction is not very sensitive and is prone to inhibition interference. For example, the normal level of oxalic acid is often below 0.23 µg/ml in plasma, but the detection limit of oxalate by OxO (e.g., Sigma Kit cat. no. 591-4) is 0.4 µg [5, 21]. Obviously, the oxalate contents in plasma are usually at a comparatively low level, lower than the detection limit of this enzymatic coupling reaction. Thus, more sensitive methods are in great need. Here immobilizing OxO and going beyond coupling reaction are to be discussed.

Immobilization of OxO

The most frequently used enzyme immobilization methods are glutaraldehyde activation and diazotization activation.

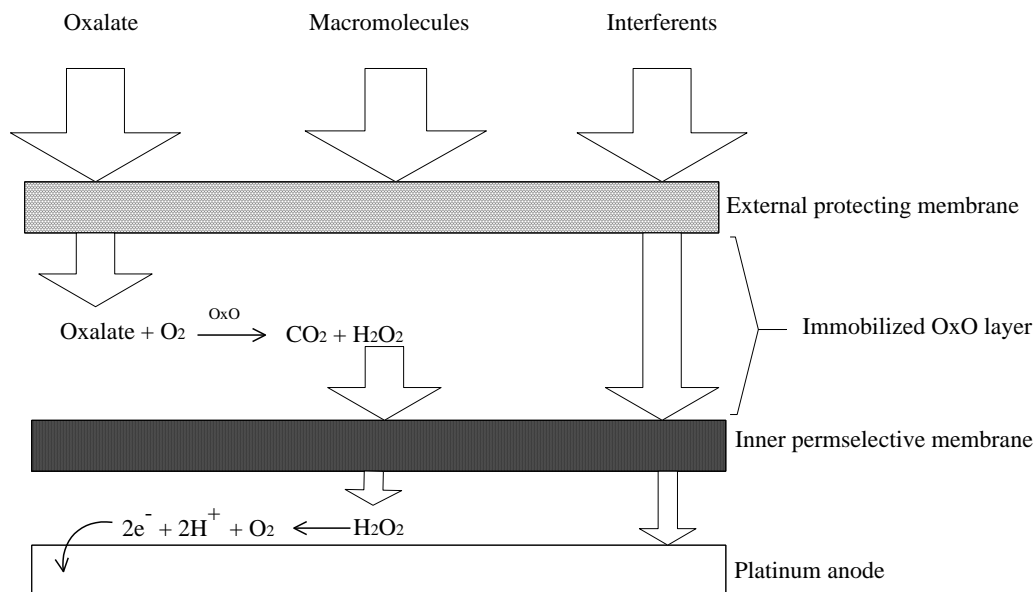


Figure 2. A typical working anode of dual-membrane amperometric OxO. The external membrane is oxalate selective and hydrogen peroxide impermeable. The inner membrane is hydrogen peroxide selective.

Glutaraldehyde is a bifunctional agent, reacting with amino groups of the carrier and OxO simultaneously to form covalent bonds. Similarly, diazo derivatives in arylamine carrier can react with amino-, imidazole-, and phenolic-groups of OxO to form covalent linkages. Chandran *et al.* [44] and Godara and Pundir [45] used the above methods to immobilize OxO to alkylamine glass beads and arylamine glass beads, respectively, to prepare for the urinary oxalate detection. Other researchers tried a variety of supporting materials, such as plasticized polyvinyl chloride (PVC) membranes [46], teflon membranes [47], silica gel [48], and Prussian blue films [21, 49], to immobilize OxO and to even build colorimetric or amperometric biosensors. Still others tried various non-traditional supporting materials for the immobilization to improve the characteristics of the enzyme. Benavidez and Baruzzi [50] immobilized OxO in mucin/chitosan hydrogels to improve the sensitivity and stability of the enzyme. Sotomayor *et al.* [51] immobilized OxO on the barley seeds powder which has sizes in the range of 20 - 35 meshes. The barley seeds have endogenous insoluble OxO, hosting the newly immobilized OxO in a native

environment, and therefore improving its stability and lifetime. Pundir *et al.* [52] used a novel supporting medium, egg shell membrane, for the immobilization of sorghum OxO. Free keto- and aldehyde groups of glycosaminoglycan on the egg shell membrane were activated and converted to amino groups with nickel and liquid ammonia, and then the activated membrane was linked covalently to the enzyme through glutaraldehyde. The approach gained a higher OxO K_m for oxalate up to 0.78 mM (the free enzyme has K_m for oxalate of 0.55 mM), but made its V_{max} for H₂O₂ 2.86-fold as compare to the free enzyme. Moreover, the immobilized enzyme exhibited a fair stability and reusability.

Oxalate detection

There are a few products commercially available on market using direct enzymatic-colorimetric method including Sigma kit for oxalate detection, ABD 3000 biosensor assay system for food oxalate analysis [53], etc. Also, Peck *et al.* [54] designed an oxalate test strip embedded with OxO, providing a qualitative method and 28 µg/ml of the limit of oxalate concentration for biological fluids. Although the

direct analysis is a simple and fast oxalate determination method with no requirement of sophisticated apparatuses, its sensitivity, specificity and anti-interference ability are relatively poor. For example, the method is not specific in measuring the oxalate contents of plant tissues or foods through the ether extraction and the calcium precipitation. In addition, the coupling reaction for hydrogen peroxide is severely interfered by inhibitors such as ascorbic acid in the extracts [55]. To overcome the limitation above, Liu *et al.* [56] and Ruan *et al.* [57] prepared insoluble OxO from wheat bran, which is like a naturally immobilized enzyme on cellulose or semi-cellulose. They applied the direct enzymatic method to analyze 120 different types of food such as lotus seeds, commonly consumed cereals, fruits, and nuts, and their results were overall in agreement with those of previously published studies.

Over the past years, a range of oxalate detection methods have been established, including titration [58], colorimetric, enzymic-colorimetric, HPLC [59], capillary electrophoresis [60], and amperometric methods. It's worth to mention that the amperometric oxalate biosensors provide a comparatively sensitive, rapid and specific method which is more suitable for routine analysis. The challenges for biosensors are instability, low conductivity and interference by electron communication. Figure 2 shows a typical amperometric oxalate biosensor of platinum anode. OxO is immobilized on a permselective membrane which allows for selective transportation of hydrogen peroxide to the electrode in the presence of interfering agents such as ascorbic acid and urate. The immobilized OxO is covered by an outer membrane which is oxalate selective and hydrogen peroxide impermeable [61]. A typical amperometric oxalate biosensor is composed of a working electrode immobilized with OxO, a reference electrode and an auxiliary electrode. Perez *et al.* [48] built a bi-enzymatic amperometric biosensor by immobilizing OxO

to silica gel on a titanium electrode as auxiliary, horse radish peroxidase on carbon paste as a working electrode, and a calomel electrode as the reference. This biosensor can detect as low as 90 μM of urine oxalate. Yadav *et al.* [62] also established a highly sensitive amperometric biosensor using the sorghum OxO for detecting the oxalate concentration as low as 3 μM . In recent years, the amperometric OxO biosensors have achieved sensitivity of detecting 1 μM of oxalate [63, 64, 65], available for plasma oxalate analysis [23].

Researchers have been developing other forms of electrode biosensors. Immobilization of OxO and catalase on barley seeds powder was tried to build a novel electrode biosensor [51]. The carbon dioxide production was measured by a carbon dioxide electrode immobilized with bromothymol blue. This method can measure oxalate as low as 8 μM . In addition, the oxygen electrode was coupled with immobilized OxO and catalase for oxalate detection [66].

The sensors based on injection of the recognition element (SIRE) systems are enzymatic amperometric biosensors. In the case of oxalate detection, OxO is often a recognition element, and an amperometric transducer device relays the signals from the enzymatic reactions to a microprocessor unit. When the OxO flows into the reaction chamber, hydrogen peroxides are released and detected by the amperometric biosensor (Figure 3). This system makes the automatic analysis of oxalate possible. Hong *et al.* [67] developed such a SIRE system for oxalate detection. In this system, OxO flew in to reach a reaction chamber, and oxalate went through a dialysis membrane to generate hydrogen peroxide which was detected with a three-electrode system. The biosensor detected oxalate as low as 20 μM . Milardović *et al.* [49] co-immobilized OxO and peroxidase on a Prussian blue film to build a novel bi-amperometric bi-enzymatic SIRE biosensor which contained two working electrodes, and the system had capacity for

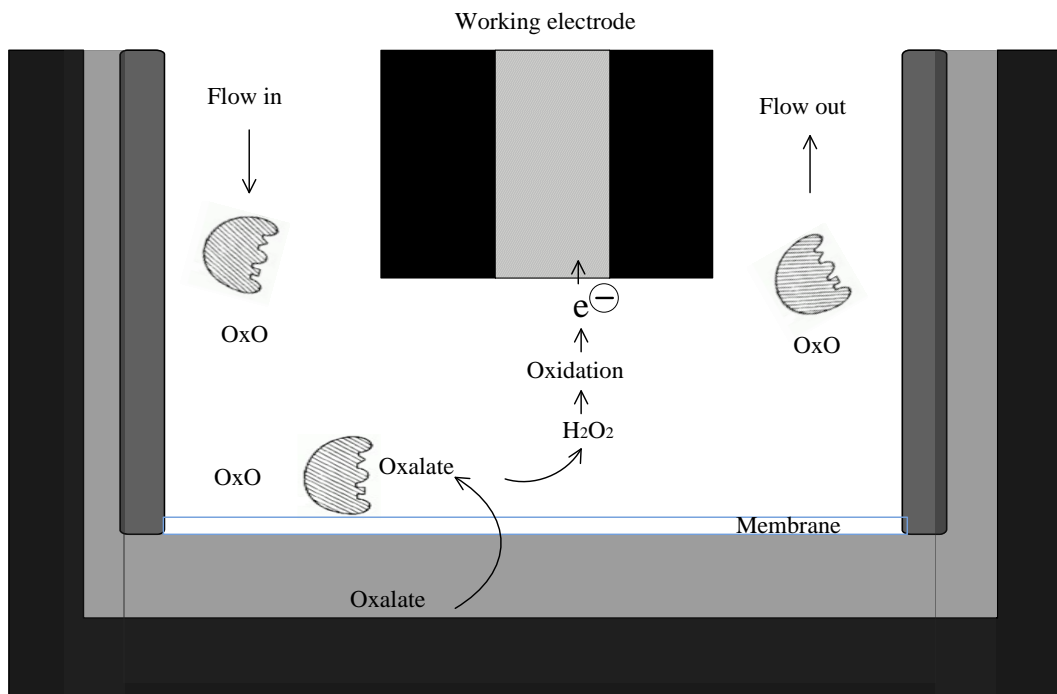


Figure 3. Schematic diagram of SIRE technology for oxalate determination. Oxalate sample gets in the reaction chamber through a membrane. OxO as the recognition element flows in the reaction chamber and generates hydrogen peroxide. Hydrogen peroxide is oxidized and detected by the working electrode.

automatic injection of oxalate samples. The detection limit reached below $1 \mu\text{M}$.

Potential medical application of OxO

Besides the oxalate detection, OxO has its application potential in medical therapies. Calcium oxalate, a prevalent encrustation mineral formed in humans, produced endogenously or ingested from food, is an end-product, which cannot be metabolized further in bodies. Calcium oxalate often causes hyperoxaluria which in turn could further result in urolithiasis. The most frequent constituent of kidney stones has been proven to be in calcium oxalate monohydrate form, which is stable thermodynamically and has a high affinity with renal tubular cells, causing oxidative stresses, cellular injuries, and cell apoptosis [68]. Due to its unique property of degrading oxalate and its unusual stability, OxO was employed as a promising auxiliary material for medical devices

in urology [69] or an enzymatic reagent for prophylactic treatment [70].

OxO in urological applications

Since Finney [71] first successfully designed a practical self-retaining silicone ureteral stent for catheters, ureteral stents have often been used to drain urine after operation. However, the mineralization of stents and jamming of catheters have become a perplexing issue for many years. The main reason for the hardening and jamming of catheters is that the accumulation and encrustation of calcium oxalate from urine [72]. Silicone elastomer is a commonly used biomaterial for urinary catheter manufacturing. Malpass *et al.* [69] immobilized OxO on the surface of silicone elastomer and conducted an *in vitro* experiment to evaluate the encrustation of oxalate calcium. The surface of silicone elastomer was aminated and linked with OxO through glutaraldehyde bioconjugation. As a result, less encrustation was deposited on catheters coated with OxO

than on the control catheters. The experiment demonstrated that immobilized OxO on coatings of indwelling medical devices was effective in lowering calcium oxalate encrustation.

Possible enzymatic supplementation therapy with OxO

Although there are some intestinal oxalate-degrading bacteria including *Oxalobacter formigenes*, *Enterococcus faecalis*, and even some strains of *Lactobacillus* and *Bifidobacterium*, oxalate in humans is a metabolic end-product with no endogenous enzyme to degrade it. With decrease of enteric colonization of these oxalate-degrading bacteria, the absorption of intestinal oxalate may increase and cause acute oxalate nephropathy [73]. Usage of OxO supplementation therapy therefore may provide a promising therapy to fight against oxalate nephropathy [70]. As it is known, OxOs from natural resources do not activate in the neutral pH environment. Raghavan and Tarachand [74] manipulated OxO made from banana fruit peels with ethylenemaleic anhydride to shift its working pH to 7.4, and then implanted into rats with the capsules containing OxO, catalase and peroxidase. The experiment demonstrated that the endogenous oxalate in rats could be decreased by the implantation of the multi-enzyme capsules and that the enzymatic therapy could be an effective solution.

Scientists and engineers have tried to explore the unique decomposing capacity of OxO and put it into practical use for therapy against oxalate related diseases including hyperoxaluria and urolithiasis. Li and Sidhu [75] embedded OxO in polymeric particle materials containing cross-linked materials, pH adjusting agents, buffering agents by oral administration to reduce the stomach oxalate. Shenoy *et al.* [76] used OxO crystals or crystals cross-linked through glutaraldehyde to reduce oxalate contents by administration orally, via upper gastrointestinal tract, or via extracorporeal

device. Other trials were by administration orally of the compositions containing bacteria generating oxalate degrading enzymes including OxO to deal with oxalate-related diseases [77, 78].

Perspective

Although much exciting progress has been made on OxO research in recent years, including the OxO 3-D structure, the elucidation of reaction mechanism, the application on enzymatic analysis for oxalate, and the medical therapy, etc., there still remain lots of problems unresolved. On large scale production, for example, the efficiency of the traditional purification protocol from wheat or barley was very low, and no practical method has been found to cleave the *N*-glycoprotein from cell walls to obtain a large quantity of soluble OxOs. Although enzymatic method for oxalate detection is very promising, there are still many challenges for researchers to design low-cost, high-sensitive, and automatic instruments.

High-speed liquid-liquid countercurrent chromatography has provided a possibility to obtain natural macromolecules to homogeneity with high efficiency and in large scale. The method overcame the low efficiency of traditional chromatography on separating biomolecules including gel filtration and affinity chromatography. For efficient separation of proteins with high molecular weight in this system, a polymer stationary phase is needed, for example, 12.5% PEG1000 is used as stationary phase to separate proteins while 12.5% potassium dihydrogen phosphate is used as a moving phase [79]. Early report demonstrated that the two liquid phase system is suitable for separation of glycoprotein [80]. Nowadays, the two key factors to control in the protein separation by the liquid-liquid countercurrent chromatography, the retention of the stationary phase and the efficient mixing of the stationary phase with the moving phase, both values of which used to be very lower in

the polymer phase system, have been promoted to a practical level. The newest report showed that the flow rate is up to 2 ml/min for separation of proteins with the liquid-liquid countercurrent chromatography [81]. Recently, our group also has been trying this technology to separate glycoproteins including OxO.

Another promising technology to get abundant large quantity of protein is simulated moving bed (SMB). SMB is a practical implementation of the continuous countercurrent chromatography, which is a separation technique simulating the solid flow by continuous switching valves. As comparison to the other traditional chromatographic methods, SMB is a continuous preparative chromatography with excellent product purity and recovery. It has been thereafter developed as a "green process" to purify active materials and is now widely used in pharmaceutical industry. It has been proved that proteins can be well separated by size exclusion SMB. For example, the maximal measured purity of the extract stream from multi-component protein mixtures, hydrolyzed casein, reached 98% [82]. Besides the size exclusion SMB, CaptureSMB, where SMB is combined with affinity chromatography [83], is a promising method for scale-up production of OxO, benefiting its specific antibody concanavalin A.

Heterologous expression is another ideal way for scale-up production of OxO. No OxO for practical use has been made from prokaryotic expression yet, but a relative enzyme particle, oxalate decarboxylase, was successfully prepared by spray-dried method expressed in *E. coli* [84]. Eukaryotic expression of heterologous OxO in *P. pastoris* is also a promising method for large scale production of OxO. Although *P. pastoris* is a good established system for production of OxO, there are still obvious disadvantages needed to be overcome, such as the severe stress on host cells by overexpression of proteins, and the possible conformational disorder by overglycosylation.

In addition, methanol used for the induction is poisonous. Thus, the new effective alternatives for the induction should be settled.

In recent years, OxO research has become a hot topic in phytochemistry owing to its value of application, and more and more patents on its practical usages have been registered and published in many countries including the United States, European countries, and China. Thus we can believe, with rapid advances in the fields of electrochemistry, biophysics, molecular biology and bioinformatics, it is possible to solve the bottleneck problem of OxO production, design highly effective recombinant OxOs and develop relative instrumentation for clinical and prophylactic therapy in the near future. We can also imagine that OxOs can have medical applications in new drug development on nephrolithiasis for its capacity of decomposing oxalate salt crystals.

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