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Investigation of epoxide hydrolase activity in *Saccharomyces cerevisiae* ORF YNR064c protein

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Investigation of epoxide hydrolase activity in *Saccharomyces cerevisiae* ORF YNR064c protein

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Abstract: The histidine-tagged variant of YNR064c protein was heterologously expressed in competent E. coli BL21 AI cells and the protein was successfully purified with immobilized metal ion affinity chromatography. The protein YNR064c displayed low epoxide hydrolase activity in a single measurement with 0.2 mM (S)-2-phenyloxirane but the activity was not reproducible.

1. Introduction

1.1. Epoxide hydrolase.

Enzymes, with the exception of catalytic RNA molecules, are functional proteins that catalyze most of the biochemical reactions in a biological system [1]. The chemical interconversions that take place inside cells, which maintain a metabolizing organism, would proceed at a very low rate without these biological catalysts. Epoxide hydrolase (EH) is an enzyme found in all types of organisms and it catalyses the conversion of epoxides (three-membered cyclic ethers) to their corresponding vicinal diols by adding a molecule of water to the epoxide ring [2] [3], *see figure 1*. The oxidative metabolism of xenobiotics (unfamiliar substances, i.e. drugs) and endogenous compound can result in epoxide intermediates which are very reactive in their nature, these molecules have a strained ring and the electronegative oxygen makes them highly polarized; the carbon atoms are therefore great targets for attack by a nucleophilic group [4]. This makes them extremely toxic because they can react with nucleic acids which are vital biological components [5]. The hydrolysis of exogenous epoxides and endogenous epoxide substrates result in products that are less reactive and easier to excrete [6].

EHs have diverse biological roles in organisms; it has been reported that EHs participate in detoxification processes, catabolism and the regulation of signaling molecules [7]. Microsomal (mEH) and soluble epoxide hydrolase (sEH) found in mammalian tissues catalyzes the hydrolysis of epoxides derived from xenobiotics as well as endogenous epoxide substrates; they play an important role in the detoxification process and differ in cellular localization and substrate selectivity [8]. Soluble EH have been shown to catalyze the hydrolysis of epoxy-eicosatrienoic acids (EETs), signaling molecules derived from essential fatty acids, which plays a role in the regulation of blood pressure and inflammation [9], [10], [11]. The enzyme has received much attention since it can be a good therapeutic target for cardiovascular diseases [12]. The biological role of plant EHs involves hydrolysis of fatty acids and biosynthetic pathway of cutin which are associated with the plants defense [13].

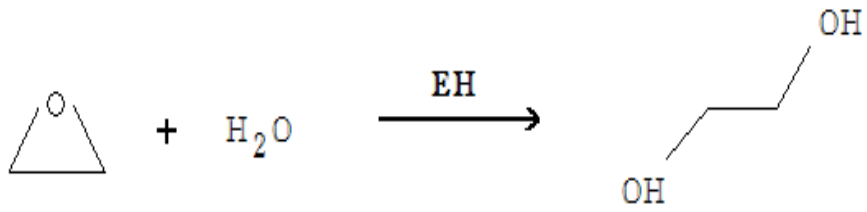


Figure 1. A simplified chemical equation of the catalytic hydrolysis of epoxide, EH adds a molecule of water to the epoxide ring forming the corresponding diol.

The biological role of microbial EHs is relatively unknown compared to animal and plant EHs, but they have gained a lot of attention because of their potential usage as biocatalysts [14].Optically pure epoxides and diols are important building blocks for the synthesis of enantiopure compound, i.e. in the production of pharmaceuticals where it is crucial to obtain high yield of enantiopure products [15], [16]. Microbial EHs show high enantioselectivty on their substrates, one enantiomer is catalyzed at a higher rate in an epoxide racemate, and have received tremendous interest over the past years because they can be utilized in the asymmetric synthesis of fine chemicals [17], [18].

1.2. Saccharomyces cerevisiae ORF YNR064c

Saccharomyces cerevisiae (baker´s yeast) is a eukaryotic model organism that has been intensively studied over the past decades; and several studies are focused on the investigation of gene products of the organism. *S. cerevisiae* alongside other yeast species and microbial organisms have been screened and examined in order to find enatioselective EHs.

The catalytic mechanism of EH involves an aspartate residue in the active site that acts as an nucleophile, a histidine that has function of a base and a carboxylate (charge-relay) adjacent to the histidine *see figure 2*. The catalytic triad residues (nucleophile, charge-relay, base) have been conserved throughout evolution and can be observed in almost all of the identified genes encoding EHs, [19]. The sequence of YNR064c was compared with genes encoding bacterial, plant and mammalian EHs, and this multiple sequence alignment showed that the residues involved in the catalytic triad (Asp nucleophile, charge-relay aspartate and His base) also reside in YNR064c, *see figure 3.*

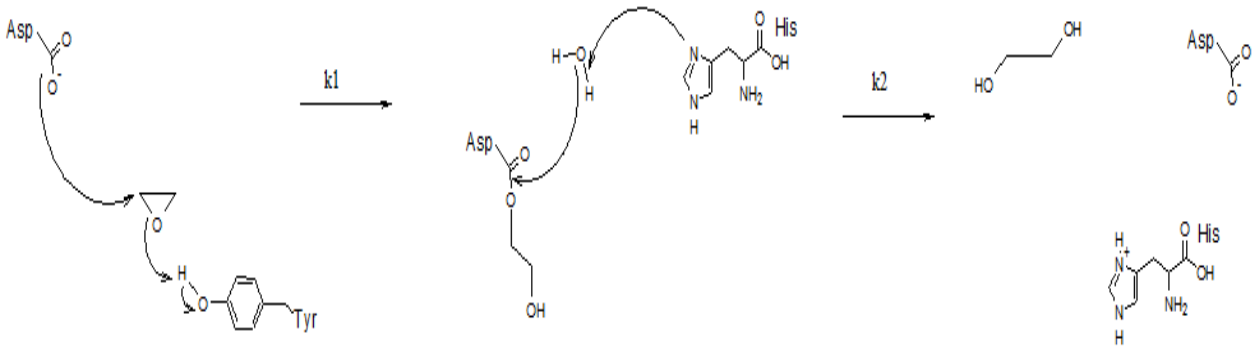


Figure 2. Enzymatic epoxide hydrolysis. The nucleophile (Nu) attacks one of the carbons in the epoxide ring, tyrosine acid (HA) assist in the opening of the epoxide ring. The alkyl-enzyme intermediate is hydrolyzed by water in the last step.

The aim of this project was to investigate epoxide hydrolase activity in ORF YNR064c protein which has been reported to display similar characteristics of an epoxide hydrolase belonging to the α/β hydrolase fold family (a broad collection of hydrolytic enzymes which includes enzymes such as dehalogenase, esterase, lipase, and peptidase). This project was based on previously investigation by Lisa T. Elfström (PhD) and Professor Mikael Widerstens "The *Saccharomyces* ORF YNR064c protein has characteristics of an 'orphaned' epoxide hydrolase", [20].When they did the experiment they experience difficulties with the expression and purification of this protein; they could not get sufficient amount of the protein YNR064c. Expression construct (pGT7YNR064c-5H) suited for the expression of this protein has been developed since then, and the general knowledge of EHs has increased tremendously over the years.

Expression of ORF YNR064c protein can be obtain by transforming a expression plasmid pGT7YNR064c-5H containing the gene into competent *Escherichia coli* BL21 AI cells, and the protein (his-tagged) can then be purified using immobilized metal ion affinity chromatography (IMAC). The molecular weight of the protein can be determined by SDS-PAGE, and functional properties like enzyme kinetics can be studied by following the hydrolysis of the substrates styrene oxide (2-phenyloxirane) in the presence of the protein, spectrophotometrically.

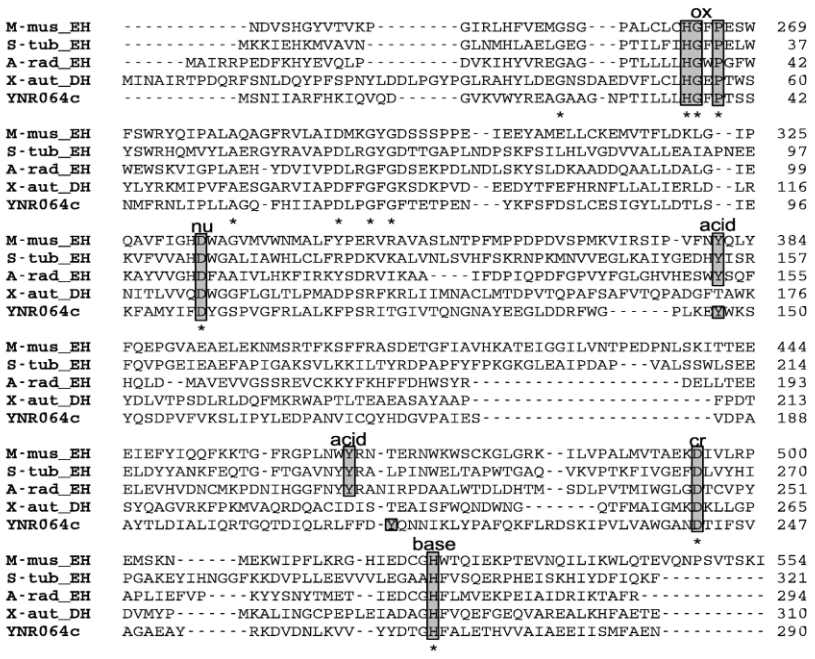


Figure 3. Sequence alignment of YNR065c with bacterial, plant and mammalian EHs and a bacterial dehalogenase. The grey areas are residues identified to be involved in the catalytic mechanism of Agrobacterium radiobacter EH, StEH1 (Solanum tuberosum) and mouse soluble EH. Residues that contributes to the formation of an oxyanion hole, which stabilizes deprotonated oxygen, are also co-aligned in YNR064c, [20].

2. Materials and methods

2.1. Heterologous expression of his-tagged ORF YNR064c protein in E.coli.

Competent BL21-AI cells (100µl) were transformed with plasmid pGT7YNR064c-5H (1µg) by heat shock transformation (42°C, ~2min). The transformed cells were then incubated for 60 min at 37°C and 200 rpm in 0.9 ml 2TY medium [1 % (w/v) tryptone, 1.6% (w/v) yeast extract, 0.5% (w/v) NaCl] prepared with 100 µg/ml ampicillin, before they were placed on LB-agar plate [1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, 1.5 % (w/v) agar] and incubated overnight.
Colonies from the overnight culture were selected for plasmid miniprep, the plasmid DNA were prepared with GeneJET^{TM} Plasmid Miniprep Kit according to the manufacturer (Thermo Fisher Scientific). Samples were sent to the Rudbeck laboratory in Uppsala for DNA sequencing.

A colony which contained the plasmid with the ORF YNR064c gene was used to inoculate an overnight culture. Approximately 35-ml culture of E.coli BL21-AI [pGT7YNR064c-5H] grown at 30°C and 200 rpm overnight in 2TY medium prepared with 100 µg/ml ampicillin were used to inoculate 3 L 2TY containing 100 µg/ml ampicillin.
The gene expression was induced with 0.2 % (w/v) L-arabinose when it had reached a cell density of OD_{600} = 1.12 and the incubation was continued for 3 h before

harvesting. The culture medium was transferred to centrifuge bottles and centrifuged at 5000 rpm for 15 min at 4 °C in a JA-14 rotor. The pellets were resuspended with approximately 20 ml of lysis buffer [buffer B (500mM NaCl, 20mM imidazol, 20mM sodium phosphate, pH 7.5), 1 tablet Complete mini, 20 µL 10mg/ml Dnase I], homogenized and cell lysates was obtained using a cell disruptor. The cell lysate (~30ml) was centrifuged for 60 min at 15 000 rpm at 4°C in a JA-25.5 rotor, and the supernatant was placed in a 50 ml falcon tube.

Samples were taken from culture induced with L-arabinose and non induced culture. Protein expression of YNR064c was then analyzed by western blot after separation by SDS-PAGE [separation gel (1.5 M Tris ph 8.8, 10% SDS, 30% acrylamide, 10% (w/v) APS), stacking gel (0.5 M Tris pH 6.8, 10% SDS, 30% acrylamide, 10% (w/v) APS)]. A total of four samples were loaded to the gel; two samples that were induced with L-arabinose and two control samples that was not induced with L-arabinose.

2.2. Protein purification

Approximately 20 ml cell lysate was added to 50 ml falcon tube containing 4.5 ml IMAC gel equilibrated with buffer B (500mM NaCl, 20mM imidazole, 20mM sodium phosphate, 0.02% sodium azide, pH 7.5) and pre-loaded with Ni(II) ions, the tube was then incubated at 4 °C on tipping table for 60 min.
The gel was centrifuged down at 700 rpm for 5 min at 4 °C and the gel were washed with 45 ml buffer W (500mM NaCl, 100mM imidazole, 20mM sodium phosphate, 0.02% sodium azide, pH 7.5). The supernatant was removed and the procedure was repeated (2x) before the gel was transferred to a 15 ml falcon tube containing 2.5 ml buffer E (500mM NaCl, 300mM imidazole, 20mM sodium phosphate , 0.02% sodium azide, pH 7.5).

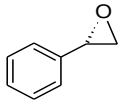
The tube which contained buffer E and the gel were incubated at 4 °C on tipping table for 20 min before it was centrifuged at 800 rpm for 5 min at 4 °C. The supernatant was removed and the procedure was repeated (1x).The final elution(2.5 ml) fraction was desalted through a PD-10 column equilibrated with 20 ml buffer D (100mM sodium phosphate, 0.02% sodium azide, pH 7.4) and the protein was eluted with 3.5 ml buffer D.

Samples(lysate, flow through, wash 1, wash 2, wash 3, elution 1 and elution 2) were collected from the purification process for an analysis by SDS-PAGE; the samples (10µl) was prepared with 10 µl sample buffer (95 µl SDS-PAGE buffer, 5 µl β-mercaptoethanol) before they were loaded together with a mix ladder [8 µl milliQ, 2 µl ladder stock (Amersham, LMW calibration kit for SDS electrophoresis 14.4 – 97 kDa, GE health care), 10 µl sample buffer] on the gel, *see figure 4* . The SDS-PAGE ran for 40 min at 200V, the gel was stained with 50 ml staining solution [25ml coomassie solution (1 tablet Phastge BlueR-coomassie, 120 ml MeOH, 80ml distilled H_2O), 25ml 20% HOAc] and destained with destaining solution (10% HAc, 40% MeOH, 50% dH_2O). The SDS-PAGE samples were heat-denatured before they were loaded on the gel.

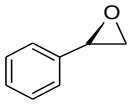
2.3. Enzyme kinetics

The initial rate of the enzymatic hydrolysis of (R)-2-phenyloxirane and (S)-2-phenyloxirane, *see figure 4*, in the presence of YNR064c protein was examined using a UV-VIS spectrophotometer. The substrates (R)-2-phenyloxirane and (S)-2-

phenyloxirane were dissolved in acetonitrile before they were diluted in 0.1 M sodium phosphate (pH 7.5, 30°C), and the final concentrations of the substrates in the cuvette (1ml) varied from 0.2mM – 1.2mM, enzyme concentration was 4.1µM in the cuvette. The hydrolysis of epoxide was followed by recording the decrease in absorbance at 225 nm.



(S) -2-phenyloxirane



(R)-2-phenyloxirane

Figure 4. The epoxide substrates used in this experiment, (S)-2-phenyloxirane and (R)-2-phenyloxirane.

Results and discussion

3.1. Heterologous expression of his-tagged ORF YNR064c protein in E.coli.

The whole sequence of the plasmid pGT7YNR064c-5H was confirmed by DNA-sequencing and expression of the recombinant YNR064c was detected by with chemiluminescense using rabbit anti-YNR064c-5H antibodies.

3.2. Protein purification

The his-tagged variant of YNR064c protein was successfully purified with IMAC. The YNR064c protein from the first batch was stored in a -20°C freezer and the protein was suspected to have lost its activity and structural integrity. Hence, another IMAC batch was made and the purified protein YNR064c was stored at +4°C.

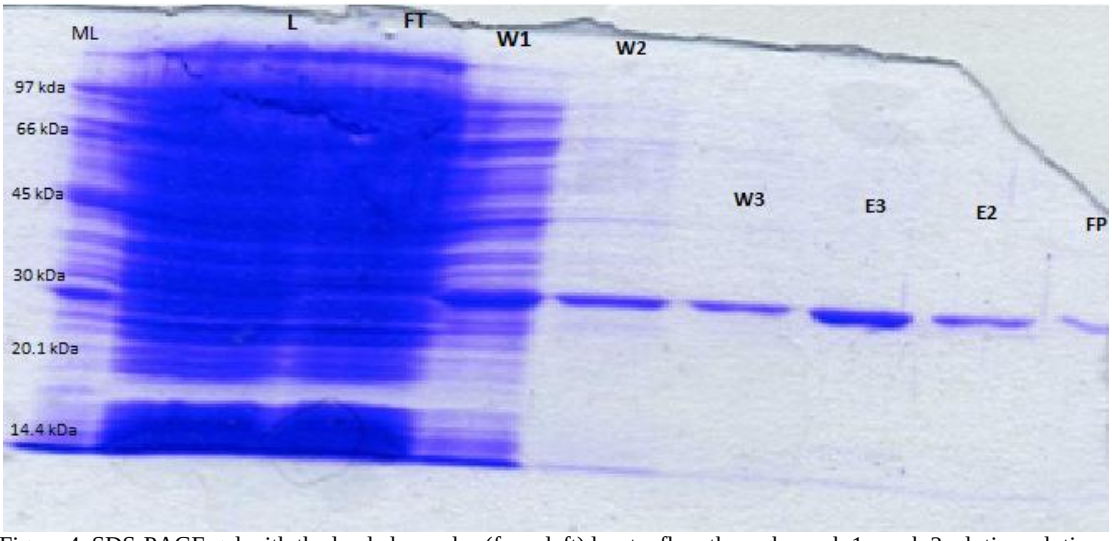


Figure 4. SDS-PAGE gel with the loaded samples (from left) lysate, flow through, wash 1, wash 2, elution, elution 2 and final product.

3.3. Enzyme kinetics

A decrease in absorbance at 225 nm which indicated activity was observed when 0.2 mM (S)-2-phenyloxirane was measured in the presence of 4.1µM YNR064c protein in 0.1 M sodium phosphate (pH 7.5, 30°C).The protein YNR064c displayed very low epoxid hydrolase activity in a single measurement with 0.2 mM (S)-2-phenyloxirane

but the activity was not reproducible, all the other measurements did not show a decrease in absorbance at 225 nm. The measurement with 0.2 mM (S)-2-phenyloxirane was among the first measurements carried out approximately 17 h after the purification process, and the remaining measurements were conducted two days after the purification. It may be that small volumes of buffer E were retained when the protein was eluted, and this has resulted in an increasing loss of activity because of the high imidazole and salt concentration. A new purification of the YNR064c protein or further investigation of epoxide hydrolase activity in YNR064c by alternative approaches such as high pressure liquid chromatography was not possible because of the limited time I had on this project

4. Conclusion and outlook

The gene product of *S. cervisiae* ORF YNR064c can be successfully expressed in competent E. coli BL21-AI cells using the expression vector pGT7YNR064c-5H and the protein YNR064c can be purified effectively with IMAC. The protein YNR064c does not show reproducible activity for the substrates (R)-2-phenyloxirane and (S)-2-phenyloxirane. I do not believe that this experiment contradict that *S. cervisiae* ORF YNR064c protein might be an epoxide hydrolase belonging to the α/β hydrolase fold family; even though this experiment did not provide any concrete evidence of epoxide hydrolase activity in YNR064c protein. It can also be that this protein has selectivity for other epoxide substrates. The tools needed for the expression and purification of this protein are not complicated and I think that further investigation of epoxide hydrolase activity in *S. cerevisiae* ORF YNR064c protein should be conducted; considering the potential application of this protein.

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