Study of three proteins in uracil catabolism pathway

Master project in Protein Science, 30 credits.

You Lv

Department of Biology, Lund University, Solvegatan 35, SE-223 62 Lund, Sweden.
Centre for Molecular Protein Science, Lund University, SE-221 00 Lund, Sweden.

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Supervisor: Professor Jure Piskur

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You Lv
Department of Biology, Lund University, Solvegatan 35, SE-223 62 Lund, Sweden.
Centre for Molecular Protein Science, Lund University, SE-221 00 Lund, Sweden.

Abstract: This study focused on expression, purification and functional characterization of three key proteins (Urc1p, Urc2p and Urc8p) in the newly discovered uracil catabolism (URC) pathway. A novel expression system was established by using *Saccharomyces kluyveri* as a host organism for expression of 6-His tagged Urc1p and Urc2p. Moreover, *URC8* gene was cloned in pET151/D-TOPO vector and expressed in *Escherichia coli*. All three recombined proteins were purified on nickel columns. The SDS-PAGE showed Urc1p and Urc8p are of correct size and high purity. This novel expression system provides an alternative or a complementary choice for eukaryotic protein production. The functional characterization assay suggested that Urc1p might open the pyrimidine ring of uridine in the URC pathway and Urc8p is a NADPH dependent reductase converting malonic semialdehyde to 3-hydroxypropionate, which is one of the final products in the URC pathway.

Key words: Uracil degradation; URC pathway; *Saccharomyces kluyveri* expression system
Abbreviations: URC, uracil catabolism; UMP, Uridine monophasphate; UDP, Uridine diphosphate; UTP, Uridine triphosphate; NADPH, dihydronicotinamide adenine dinuclectide phosphate; NADH, nicotinamide-adenine dinucleotid; IPTG, Isopropyl-β-D-thiogalactoside; KP buffer, potassium phosphate buffer.

Supervisor: Jure Piskur Department of Biology, Lund University, Solvegatan 35, SE-223 62 Lund, Sweden. Fax: +46 46 2224113. Tel: +46 46 2228373. E-mail: jure.piskur@cob.lu.se.

1. Introduction

Pyrimidine bases are central precursors in cells and they are the basic blocks for building DNA and RNA. Their intracellular pools are tightly balanced by *de novo* biosynthesis, salvage and degradation pathways [1-4]. Understanding pyrimidine degradation is significant as it involves human health for example degradation of the anticancer drug 5-fluorouracil [5]. It is also extremely useful for our understanding of the evolution of life [6]. Three pyrimidine degradation pathways, namely the Reductive, Oxidative and Rut pathways, were discovered before [4, 7]. Using yeast, *Saccharomyces kluyveri* (also named *Lachancea kluyveri*), as a model organism, another novel pyrimidine degradation pathway named uracil catabolism (URC) pathway was discovered by our group [8].

Eight genes, named *URC1-8* are encoding corresponding proteins (Urc1-8p) in this novel catabolic pathway [8, 9]. Urc2p is a transcription factor which regulates genes transcription in this pathway and Urc7p is a transporter. Urc3,5p which is homologous to Dur1,2p found in *Saccharomyces cerevisiae* (baker's yeast) was indentified as urea amidolyase. The *URC6* gene,

which is close to the *URC1* and *URC4* gene in the genome, is predicted to encode uracil phosphoribosyltranferase. Urc1p and Urc4p are highly conserved proteins widely distributed in various prokaryotes and fungus. In the pathway, uracil is finally converted to ammonia, carbon dioxide and 3-hydroxypropionic acid; while UMP, ribosylurea and urea are considered as intermediates [8, 10] (Figure 1). Despite the bioinformatics predictions, functions of Urc1p, Urc4p and Urc8p still remained a mystery and our knowledge of the URC pathway is, at best, very incomplete.

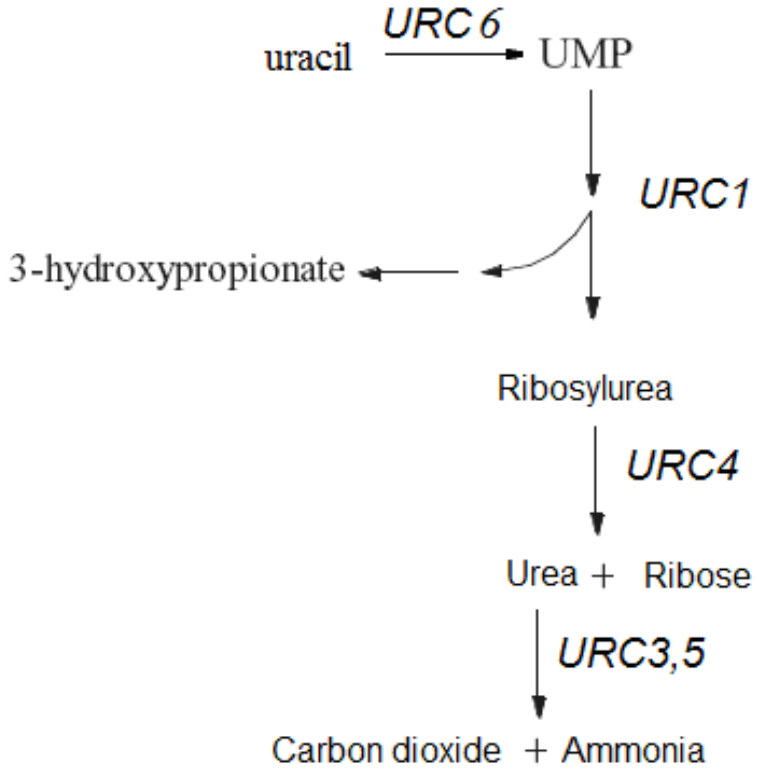

Figure1. The proposed URC pathway based on previous study [8, 10]. The pathway begins with degradation of UMP. First, uracil is converted to UMP by uracil phosphoribosyltransferase encoded by *URC6* gene. In the second step, the pyrimidine ring of uridine is opened by Urc1p and release ribosylurea which is accumulated in *URC4* mutants and an unknown intermediate which is finally converted to 3-hydroxypropionate. In the third step, ribosyurea is converted to urea and ribose by Urc4p. Finally, Urea is catabolized by Urc3,5p and generates carbon dioxide and ammonia which is a useful nitrogen source.

Yeasts are unicellular eukaryotic microorganisms classified in the kingdom of fungi. Apart from their applications in baking industry and alcoholic industry, some yeasts such as *S. cerevisiae* and *S. kluyveri* are used as important eukaryotic model organisms for cell biology and genetic studies [6, 11-13]. Their eukaryotic features also make them favorable hosts for eukaryotic proteins expression, especially many proteins which can not fold correctly in *Escherichia coli* expression system. In recent years, several yeast expression systems such as *S. cerevisiae*, *Kluyveromyces lactis* and *Pichia pastoris* expression systems have been developed for expression of a variety of heterologous proteins [14].

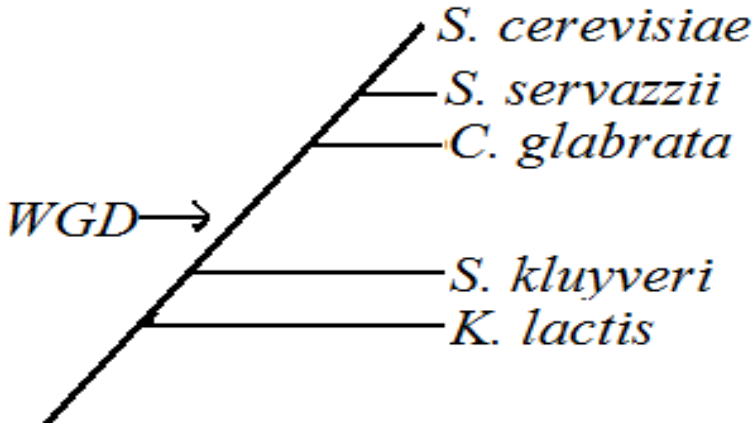

Figure 2. A simplified phylogenetic tree of 5 prominent yeast species and the occurrence of the whole genome duplication (WGD), which took place approximately 100 million years ago. So *S. cerevisiae* and *S. kluyveri* are not very closely related.

S. kluyveri is a budding yeast isolated within the yeast flora from the intestinal canal of Drosophila in the Yosemite region, California in 1956 [15]. It separated evolutionarily from *S. cerevisiae* over 100 million years ago before the whole genome duplication event [6, 16] (Figure 2). As a nonconventional yeast, *S. kluyveri* is used as a model organism to study pyrimidine metabolism [8, 12, 13]. However, using *S. kluyveri* as a host for protein production has not been reported yet. Due to the unsuccessful expression of Urc1p and Urc2p in conventional expression systems, both *E. coli* and yeast expression systems, a novel expression system was established. A universally used yeast/ *E. coli* shuttle plasmid P300 (also named YEp352) which was modified by adding a *S. kluyveri TPI1* promoter **(**Triosephosphate isomerise 1 promoter**)** in the multiple cloning site, was used as the expression vector and *S. kluyveri* was used as the expression host. Moreover, the *URC8* gene was cloned and expressed in *E coli* BL21 Star™ cells. These three proteins were subsequently purified on nickel columns. In an attempt to elucidate the URC pathway, functional characterization assay of Urc1p and Urc8p was performed.

2. Materials and methods

2.1. Reagents and enzymes

Protein markers, DNA ladders, T4 DNA ligase, DNA polymerase, Alkaline Phosphatase, restriction enzymes, PCR purification kit, gel purification kit and plasmid purification kit were purchased from Fermentas. NADH and NADPH were purchased from Sigma. Ni Sepharose and PD 10 columns were purchased from GE healthcare. Yeast nitrogen base (YNB) without $(NH_4)_2SO_4$ and Uracil drop out were purchased from Formedium. Complete EDTA-free Protease Inhibitor was purchased from Roche.

2.2. Medium, plates and buffers

LB medium: 1liter medium contains 5g yeast extract, 10g peptone, 10g NaCl and 100mg ampicillin.

YPD medium: 1liter medium contains 5g yeast extract, 10g peptone, 20g glucose.
SD medium: 1liter medium contains 5g succinic acid, 3g NaOH, 1.7g YNB without $(NH_4)_2SO_4$ and 5g $(NH_4)_2SO_4$.
SC medium: 1liter medium contains 5g succinic acid, 3g NaOH, 1.7g YNB without $(NH_4)_2SO_4$, 5g $(NH_4)_2SO_4$ and 2g Uracil dropout.
Uracil medium: 1liter medium contains 5g succinic acid, 3g NaOH, 1.7g YNB $(NH_4)_2SO_4$ and 1g uracil.
20g agar was used in above mediums when making their corresponding plates.

25/50 mM DTT/KP buffer: 10ml buffer contains 2.5ml 0.1M DTT, 1ml 1M KP buffer (pH7.4) and 6.5ml sterilized water.
STM buffer: 270mM Sucrose, 10mM Tris-HCl (pH 7.5) and 1mM $MgCl_2$.
His binding buffer: 50mM imidazole, 20mM Tris-HCl(pH7.5) and 500mM NaCl.
His elution buffer: 500mM imidzole, 20mM Tris-HCl(pH7.5) and 500mM NaCl.

2.3. Primers, plasmids and strains

Table 1. Primers

Designation	Primer sequence (5'→3')
prTPI1 EcoRI fw	GGAATTCATTCACCACACCCATCTAC
prTPI1 SacI bw	CGAGCTCTGTGTGTATTTTATTTGAGTTAGAG
Sk URC1 KpnI fw	CTGGTACCATGTCCCCTATTGCTGTTACCTC
Sk URC1 6His SalI bw	GCGGTCGACTTAATGGTGATGGTGATGATGTTTGACGTCATTCCACGTT
Sk URC2 (ATG)2 KpnI fw	GCTGGTACCATGGCCTCCGACAACAG
Sk URC2 6His SphI bw2	GACATGCATGCTTAATGGTGATGGTGATGATGCTGCTGCTGCTGCTGCA
P300 fw	TGTGGAATTGTGAGCGGATA
P300 bw	GTTTTCCCAGTCACGACGTT
URC8 TOPO fw	CACCTCAAGGTAGAAGGGCTGC
URC8 TOPO bw	TTAGTCACCGCGATAGATATGG
T7	TAATACGACTCACTATAGGG

The DNA binding part of the primer was underlined.

Table 2. Plasmids

Designation	Reference/ origin	Features
P300	YEp352	The same as YEp352
P1018	P300	With *S. kluyveri TPI1* promoter
P102X1	P1018	With 6-His tagged *URC1* gene
P102X2	P1018	With 6-His tagged *URC2* gene
pET151/D-TOPO		
P102X3	pET151/D-TOPO	With *URC8* gene
P895	pET151/D-TOPO	With *PYD4* gene

Plasmids with *URC1* gene or *URC2* gene cloning have not been given a new P number yet. So the

name of P102X plus a number is given temporarily.

TOP10 *E. coli* strain was used for plasmid preparation and BL21 Star™ *E. coli* strain was used for Urc8p and Pyd4p expression. The yeast strains are listed in table 3.

Table 3. *S. kluyveri* strains

Designation	Reference/ origin	Genotype	Comments
Y156	J.Strathern,GRY1175	*MATα ura3*	
Y1156	Y90	*MATα thr urc1::KanMX3*	Deletion
Y116X1	Y156	*Tansformed with P102X1 plasmid*	
Y116X2	Y156	*Tansformed with P102X2 plasmid*	
Y116X3	Y1156	*Tansformed with P102X1 plasmid*	

Strains transformed with P102X1or P102X2 plasmid have not been given a new Y number yet. So the name of Y116X plus a number is given temporarily.

2.4. Expression and purification of Urc1p and Urc2p; and functional characterization of Urc1p

2.4.1. Expression vector construction

prTPI1 EcoRI fw and prTPI1 SacI bw were used for cloning of *S. kluyveri TPI1* promoter. The PCR product was purified by PCR purification kit and subsequently digested by EcoRI and SacI. The P300 plasmid was digested by EcoRI and SacI at 37°C for 15 min, and then 1µl fast digest Alkaline Phosphatase was added for 10min incubation to remove the 5' end phosphate of the digested plasmid. The digested plasmid was purified according to Fermentas gel purification kit. T4 DNA ligase was used for the ligation of the digested plasmid (54ng) and *TPI1* gene (15ng) in a 20µl reaction volume. Followed by the ligation, 2µl of DNA (plasmid with insert) was transformed into *E. coli* TOP10 cells via heat shock transformation method and the plate was incubated overnight at 37°C. The newly constructed plasmid with the *TPI1* promoter was confirmed by colony PCR and gel electrophoresis to make sure the gene was in the vector. Sequencing was performed to confirm the DNA section with *TPI1* promoter. The newly generated plasmid was given the name of P1018 (Table 2).

SkURC1 KpnI fw primer and SkURC1 6His SalI bw were used for the *URC1* gene cloning and the PCR product of *URC1* gene was purified and digested with KpnI and SalI. The *URC1* gene was then ligated in P1018 and transformed into *E. coli* TOP10 cells. The gene was then confirmed with colony PCR, gel electrophoresis and sequencing. The newly constructed plasmid with the *URC1* gene was given the name of P102X1 (Table2).

Sk URC2 (ATG)2 KpnI fw primer and Sk URC2 6His SphI bw2 primer were used for cloning of *URC2* gene. The construction of expression vector for Urc2p was similar to Urc1p expression vector construction. The newly constructed plasmid with *URC2* gene was given the name of P102X2 (Table2).

2.4.2. Transformation of *URC1* gene and *URC2* gene in *S. kluyveri*

2ml of *S. kluyveri* competent cells (OD600 between 0.8 and 1.2 in YPD medium) was harvested (3500rpm, 5min) and washed with STM buffer. Cells were resuspended with fresh 25/50mM DTT/KP buffer and incubated at 37°C for 30min. Whereafter, cells were transferred on the ice and spined down (3500rpm, 5min, +4°C). Then, cells were washed with 2ml ice-cold STM buffer twice and resuspened in 100µl ice-cold STM buffer. About 200ng DNA (volume less than 10µl) was used for transformation and the transformation was carried out via electroporation (the transformation condition is 1.5kV, 100Ω, 25µF and 1.6 to 2.1 seconds). After transformation, 800µl YPD medium was added to the cells, followed by incubation for 1 hour at 25°C. Finally, cells were concentrated to 100µl and plated on SD plates. The newly generated two strains were named Y116X1 (with P102X1 plasmid) and Y116X2 (with P102X2 plasmid) (Table 3).

2.4.3. Functionality check of Urc1p

The *URC1*gene knockout strain Y1156 can not use uracil as sole nitrogen source because the pathway for utilization of uracil was blocked. The P102X1 plasmid was transformed into Y1156 cells and the cells were plated on uracil plates. The new generated strain was given the name of Y116X3. If the Y116X3 cells can grow on uricil plate, it indicates that 6-His tagged Urc1p was expressed, correctly folded and can carry its function in Y1156.

2.4.4. Purification of 6-His tagged Urc1p and Urc2p

The Y116X1 strain and Y116X2 strain were cultured in SC medium. 1.5 liter of cell culture with OD 600nm of 2.5 to 3.5 was harvested and resuspend in 40ml His binding buffer plus 1 tablet of Complete EDTA-free Protease Inhibitor. Cells were disrupted twice by French press with pressure of 1300 psi. The liquid with cell debris was centrifuged at 10000 rpm for 30min and the supernatant was used for protein purification. The purification was carried out on 1ml nickel column with the force of gravity. After flow through of the supernatant, the column was washed with 20ml of His binding buffer. 10ml His elution buffer was used for protein elution and different factions (12 drops per faction) were collected in 1.5ml Eppendorf tubes.

The protein concentration was measured using the Bradford assay. The purity of Urc1p and Urc2p were analyzed by SDS-PAGE with Coomassie Brilliant Blue staining and silver staining, respectively.

2.4.5. Functional characterization of Urc1p

Uracil, uridine, UMP, UDP or UTP solution with concentration of 20mM was used as potential substrate of Urc1p. Briefly, 1µl to 5µl of uracil, uridine, UMP, UDP or UTP was added into 975µl of phosphate buffer (pH 7.4) or Tris-HCl buffer (pH 6.8). The UV absorption of the solution at 260nm was adjusted to between 0.5 to 0.8. Then, 20µl of purified Urc1p with or without imidazole was added into the cuvette to observe to the decrease in absorption at 260nm (the absorption of pyrimidine ring) at room temperature. To test if the Urc1p functions when it interacts with another protein partner, Urc1p purified from Y115X3 strain was used for the assay. The bioinformatics presiction suggests that Urc1p is a Mg^{2+} and Zn^{2+} binding protein. Because imidazole can interact with Zn^{2+}, the Zn^{2+} in the protein may

bind with imidazole during the protein purification and the activity thus may be inhibited. As a result, 0.5ml 1M $ZnCl_2$ and $MgCl_2$ solution was added into 0.5ml of Urc1p fraction to compete with the imidazole which might bind on the Zn^{2+} in the protein.

2.5. Expression, purification and functional characterization of Urc8p

2.5.1. Expression of Urc8p

URC8 TOPO fw primer and URC8 TOPO bw primer were used for cloning of the *URC8* gene. The purified PCR product was inserted into pET151/D-TOPO plasmid by TOPO cloning and a new plasmid named P102X3 was generated (Table 2). The P102X3 plasmid was transformed into *E. coli* TOP10 cells for plasmid preparation. The expression vector was analyzed by colony PCR and gel electrophoresis. The section with the *URC8* gene in plasmid P102X3 was sent for sequencing and T7 primer was used as sequencing primer. The plasmid with *URC8* gene was then transformed into *E. coli* BL21 Star™ cells for Urc8p expression. Cells were grown in 2 liter of LB medium with 0.1mg/l ampicillin at 37°C until OD600 was around 0.6. Followed by induction with IPTG, the culture was incubated at 16°C overnight for Urc8p expression.

2.5.2. Purification of Urc8p

The cell culture for Urc8p production was harvested and resuspend in 40ml of His binding buffer plus 1 tablet of Complete EDTA-free Protease Inhibitor. Cells were disrupted by French press with the pressure of 1000 psi twice. The purification was performed on 6ml nickel column. The purification procedures were similar to Urc1p and Urc2p purification described in 2.4.4 excepting different washing and elution volumes.

2.5.3. Expression and purification of Pyd4p

The vector for Pyd4p expression was constructed previously in our lab. The purification procedures were the same as the purification of Urc8p.

2.5.4. Functional characterization of Urc8p

Pyd4p was used to convert β-alanine and α-ketoglutarate to malonic semialdehyde. Before the assay, Urc8p and Pyd4p were run on a PD-10 column with 0.1M KP buffer containing 10μM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (pH7.4) and 50mM Tris-HCl buffer plus 100μl Pyridoxal phosphate (the cofactor) respectively to remove salts and imidazole. 780μl 0.1M KP buffer (pH7.4), 100μl 0.76M β-alanine, 50μl Pyd4p (1.3mg/ml) and 20μl 0.25M α-ketoglutarate were added to the cuvette for a 5min pre-incubation at 30°C. Afterwards, 20μl NADPH or NADH and 10μl Urc8p (0.03mg/ml) was added and the absorption at 340nm (the absorption of NADPH or NADH) was monitored.

3. Results

3.1. The expression vectors were successfully constructed

A new plasmid, named P1018, was generated with *TPI1* promoter in front of multi-cloning

site. The 6-His tagged *URC1* gene and *URC2* gene were cloned in P1018 plasmid generating two new plasmids named P102X1 and P102X2, respectively. The sections of foreign genes (*TPI1* promoter, *URC1* gene and *URC2* gene) in the plasmids were confirmed by sequencing. A series of new plasmids with expected sequence and features were successfully constructed (Figure 3, Table 2). The Y116X3 strain can grow on uracil plates, indicating that 6-His tagged Urc1p can fold correctly and carry out its function in *URC1* gene knock out *S. kluyveri*. The sequencing results also demonstrated that the plasmid for Urc8p expression (P102X3) was successfully constructed (Table 2).

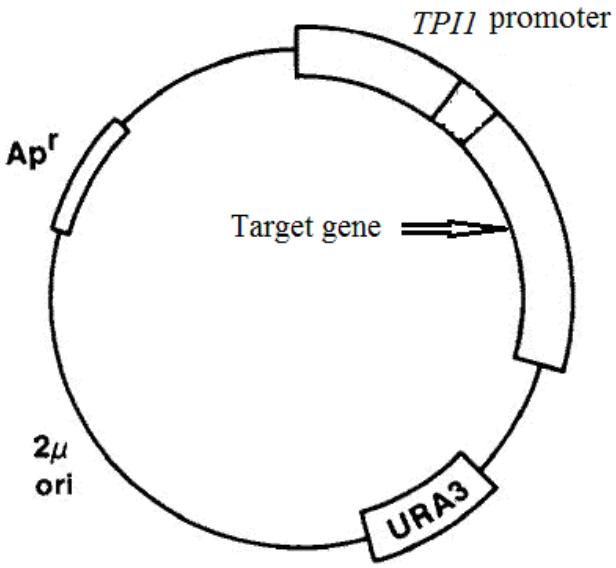

Figure 3. The schematic diagram of newly constructed plasmids (P1018, P102X1 and P10X2).

3.2. Three proteins were expressed and purified

The single band with expected size on the SDS-PAGE image shows that Urc1p and Urc8p were successfully expressed and purified (Figure 4a and b). The yield of Urc1p and Urc8p were 1.5mg and 10mg per liter, respectively. The SDS-PAGE image of Urc2p shows that Urc2p was expressed and purified, however degraded because there are many bands with the molecular weight the same or smaller than Urc2p (83kDa) (Figure 4c).

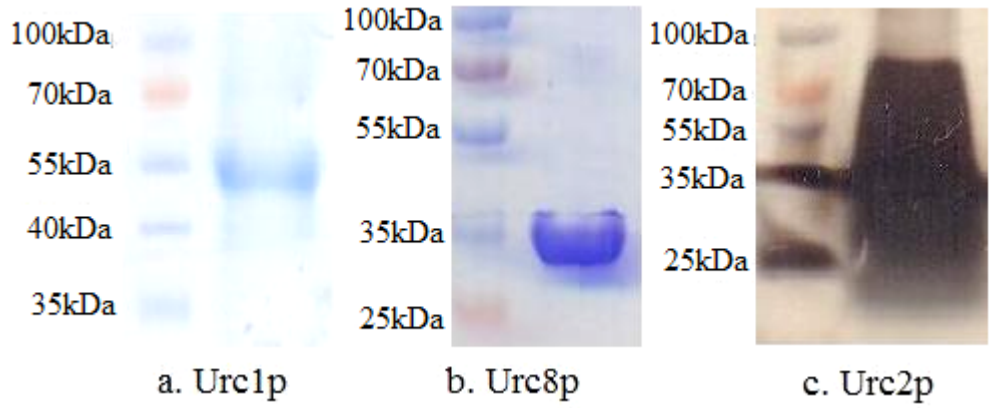

Figure 4. The SDS-PAGE pictures of Urc1p (a), Urc8p (b) and Urc2p (c). The calculated molecular weight of 6-His tagged Urc1p, Urc8p and Urc2p are 50kDa, 33kDa and 83kDa, respectively.

3.3. Urc1p may open the pyrimidine ring of uridine

The Urc1p from Y116X1 strain both with and without imidazole did not exhibit any activity in opening the pyrimidine ring of any potential substrates. However, a decrease in absorption at 260nm (the absorption of pyrimidine ring) resulting from opening of pyrimidine ring was observed when uridine was used as substrate and Urc1p purified from Y116X3 strain was used to catalyze the reaction. In such reaction the 0.5ml Urc1p solution (concentration is too low to be measured) was pretreated by adding 0.5ml solution containing 1M $ZnCl_2$ and $MgCl_2$. The deposition was removed subsequently and the supernatant was used for the reaction. This experiment suggested that the decrease in absorption was caused by opening of the pyrimidine ring and uridine is the substrate.

3.4. Urc8p is a NADPH dependent reductase for malonic semialdehyde

A linear decrease in absorption at 340nm (the absorption of NADPH or NADH) from 0.825 to 0.494 was observed during 10minutes when NADPH was used as in the reaction. No change of absorption at 340nm was observed when NADH was used in the reaction. The experiment demonstrated that the NADPH was depleted during the reaction and the malonic semialdehyde which converted by Pyd4 was reduced simultaneously.

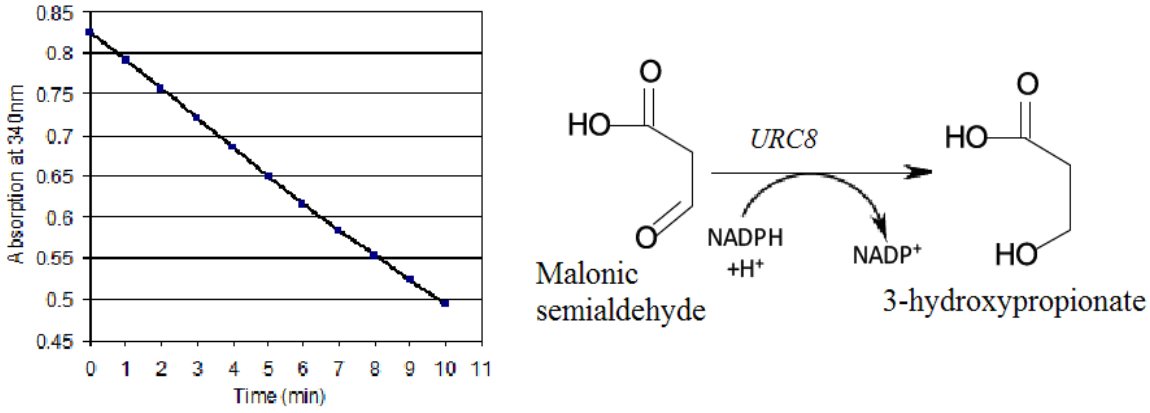

Figure 7. The figure on the left demonstrates the monitoring of absorption at 340nm every minute. The figure on the right shows the reaction catalyzed by Urc8p. The malonic semialdehyde was reduced to 3-hydroxypropionate if NADPH was present.

4. Discussion

Yeasts have been commonly used as the host cells for expression of heterologous proteins for 3 decades since onset of gene technology. The development of expression system, at the beginning, was focused on the best characterized species such as *S. cerevisiae.* However, some alternative yeast hosts such as *Kluyveromyces lactis, Hansenula polymorpha* and *Pichia pastoris* have been proven to offer significant advantages over the traditional baker's yeast for the production of certain proteins [17]. Consequently, the exploration of new yeast expression systems which exhibit superior features than existed systems is significant, and the potential is great because there are thousands of different yeasts on our planet. Nevertheless, the insertion

of a foreign gene into an expression vector and transformation of such vector does not guarantee a successful expression of foreign protein since numerous problems may arise in this multi-step process. Particularly, the choice of promoter and expression plasmid, which has decisive effects on the quantity and quality of foreign protein production in cells, is extremely important and challenging [14, 17]. In the present investigation, the common used yeast/ *E. coli* shuttle plasmid P300 was modified by inserting *TPI1* promoter as the expression vector. Therefore, the new plasmid (P1018) inherits all the features of P300 plasmid, such as a uracil-selectable marker for transformation (*URA3* gene), a 2μ *ori* and ampicillin resistance gene, which were resulting an easy manipulation at molecular genetic level. A strong promoter *TPI1* promoter, which is homolog to *S. cerevisiae TPI1promoter,* was introduced into this system, might give a highly gene expression on mRNA level. The expression of Urc1p with high yield and a soluble form showed advantages of present system. In contrast, the expression of Urc2p was not as successful as Urc1p. Instead of a single band, there were lots of bands appearing on the SDS-PAGE with molecular weight the same or lower than Urc2p. Noticing Urc2p is a transcription factor, these proteins may be resulting from the degradation because transcription factors normally have shorter half-life.

The previous studies on the URC pathway in our group suggest uracil or uridine is converted to UMP by Urc6p or uridine kinase, respectively, before the degradation, and 3-hydroxypropionate is one of the final products [8, 10]. In the present study, a decrease in absorption at 260nm was observed when uridine was used as substrates indicates that the degradation begin with uridine, not UMP. Moreover, previous study in our group found that ribosylurea, not phosphate rabisylurea, accumulated in urc4 mutants strain. Such findings also support the hypothesis that uridine might be the first substrate in the URC pathway [8]. In addition, it is not economical for cells spending energy to convert uracil or uridine to UMP then degrade it rather than degrade uridine directly. But why would the degradation start with uridine and not uracil? The author suggests that adding a polar ribose molecular on less-polar uracil may prevent lose of uracil from non-polar cell membrane. Therefore, uridine might be opened in both C4-N3 bond and C6-N1 bond, and release one molecular of malonic semialdehyde and one molecular of rabisylurea. The author also notices that reaction was only observed when Urc1p from *URC1* knockout strain (Y116X3) which was cultured in uracil medium. Before the reaction, 0.5ml 1M $ZnCl_2$ and $MgCl_2$ solution was added in to 0.5ml Urc1p solution. Therefore, the author deduces that Urc1p may be functional by interacting with another protein partner and imidazole may inhibit the reaction by binding with Zn^{2+} in Urc1p. However, more investigation is needed to prove such hypothesis and the experiment also needs to be repeated. During the Urc8p activity assay, a decrease in absorption at 340nm was observed when NADPH, not NADH, was used as reducing agent. The substrate used in the assay was malonic semialdehyde, which was produced by Pyd4p, then subsequently reduced to 3-hydroxypropionate. Such conclusion is corresponding to the fact that 3-hydroxypropionate is one of the final products in the URC pathway. Finally, a proposed process for the URC pathway based on the present study and current knowledge on the URC pathway was presented (Figure 8).

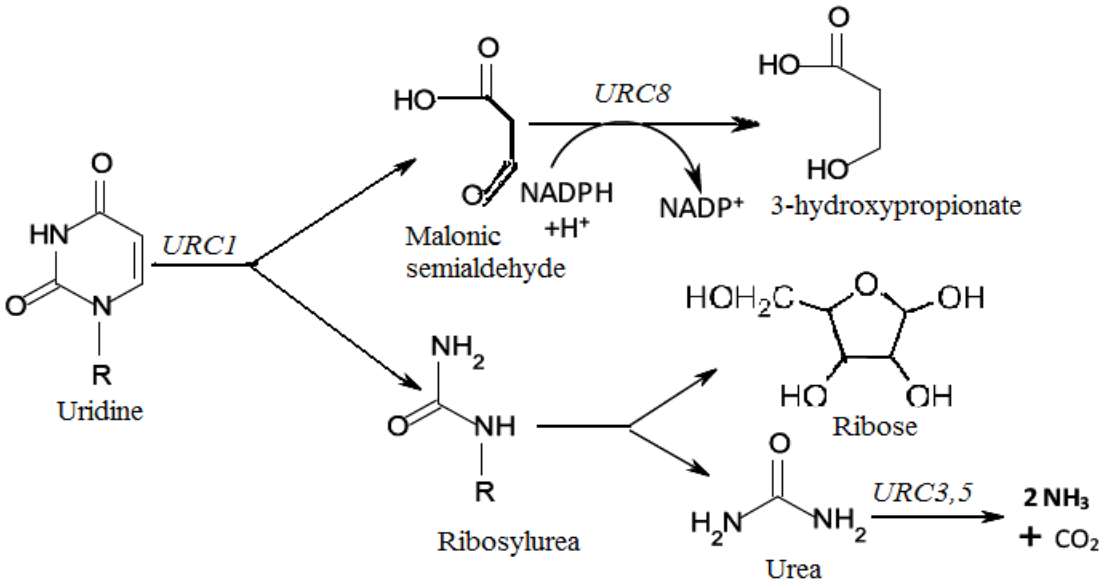

Figure 8. The proposed URC pathway (R=ribose group). The pathway starts with opening of uridine in both C4-N3 bond and C6-N1 bond by Urc1p or Urc1p with its partner protein, and release one molecule of malonic semialdehyde and one molecule of ribosylurea. The malonic semialdehyde is subsequently converted to 3-hydroxypropionate which is one of the final products in the pathway. Ribosylurea is converted to ribose and urea by unknown protein (might be Urc4p). However, the author tested Urc4p and failed to observe any activity (data not shown). So this step is still mysterious. Finally, urea is catabolysed by Urc3,5p and releases 2 molecule of ammonia and one molecule of carbon dioxide.

In conclusion, a novel *S. kluyveri* protein expression system was established for the first time to express Urc1p and Urc2p. Such system is simple and efficient; and provides an alternative or a complementary choice concerning expression eukaryotic proteins in yeasts. The elucidation of the function of Urc8p and the assay of Urc1p give us a deeper understanding of catalytic reaction in the URC pathway as well as uracil metabolism in other organisms with similar or the same pathway.

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