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Expression of Human Heavy-Chain and Light-Chain Ferritins in *Saccharomyces cerevisiae* for Functional Foods and Feeds

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To produce human ferritins in yeast, human H-chain and L-chain ferritins were amplified from previously cloned vectors. Each amplified ferritin gene was inserted into the pYES2.1/V5-His-TOPO yeast expression vector under the control of the GAL1 promoter. Western blot analysis of the recombinant yeast cells revealed that H-and L-chain subunits of human ferritin were expressed in *Saccharomyces cerevisiae*. Atomic absorption spectrometry (AAS) analysis demonstrated that the intracellular content of iron in the ferritin transformant was 1.6 to 1.8-fold higher than that of the control strain. Ferritin transformants could potentially supply iron-fortified nutrients for food and feed.

Key words: Atomic absorption spectrometry, human ferritin, iron, *Saccharomyces cerevisiae*

Introduction

Iron is one of the essential inorganic elements found in mammalian, plant, and bacterial cells. Trace minerals such as iron, zinc, copper, selenium, manganese, etc, are essential for metabolism but toxic at higher amounts in the body. Ferritin, an iron storage protein composed of 24 subunits of globular structure, has an important role in iron storage, iron detoxification and maintaining iron in a metabolically accessible form [15].

In mammals, two types of subunits, H (heavy) and L (light) polypeptides, have over a 50% amino acid sequence homology and are found in varying ratios in ferritin from different tissues [5, 23]. H- and L-ferritins store iron atoms in a central cavity in the form of a hydrous ferric oxide mineral core [3]. The H-ferritin can induce a rapid oxidation of iron from Fe (II) to Fe (III) due to the presence of a ferroxidase center located in the inner portion of the subunit fold [11, 12, 21]. Whereas, the L-ferritin has a higher efficiency in accumulating iron mineralization within the cavity and is more stable from physical denaturation than H-ferritin [13, 21].

A variety of recombinant ferritins in mouse, rat, bovine, tadpole, horse, and humans have been reported and expressed in *E. coli*, insect cells, and yeast [1, 4, 13, 16, 17, 18, 20]

In our previous reports, human H-ferritin [7, 9] and L-ferritin [8, 9] were successfully expressed in the yeast *Pichia pastoris*. In this study, two kinds of H- and L-ferritin were amplified by the PCR and constructed with an *S. cerevisiae* expression vector to produce recombinant ferritins in transformants. The purpose of this study was to achieve expression of recombinant human ferritins in *S. cerevisiae* and to determine which type of ferritin transformant results in greater iron storage *in vivo*. *Saccharomyces cerevisiae* is generally recognized as a safe (GRAS) organism and has been used for the production of valuable proteins. A similar strategy has value in expressing food-grade ferritins to be used in the design of functional food ingredients such as, special purpose yeast products, yeast cultures, nutritional yeast products and probiotics.

Materials and Methods

Vector Construction

The pYES2.1/V5-His-TOPO plasmid (Invitrogen, Carlsbad, CA, U.S.A.) contains a uracil (URA3) marker, a galactokinase (GAL1) promoter and a topoisomerase-cloning site. Human H- and L-ferritin genes were amplified by the PCR using ferritin-cloned vectors (pPICH-Fe and

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pPICL-Fe) as templates [7, 8]. The primers for cloning were based on the human H- and L-ferritin sequence from Genbank (Accession no. M97164 and M11147) and were commercially synthesized (Bioneer, Taejon, Korea). The primer sequences were as follows: S.CH-Fe5: 5'-CAG CTA TGA CGA CCG CGT CCA CCT CGC AGG -3'; S.CH-Fe3: 5'-GCT TTC ATT ATC ACT GTC TCC CAG GGT GTG -3'; S.CL-Fe5: 5'-CAG CTA TGA GCT CCC AGA TTC GTC AGA ATT -3'; and S.CL-Fe3: 5'-GTC GTG CTT GAG AGT GAG CCT TTC GAA GAG -3'. The PCR used a first denaturation step at 94°C for 3 min 30 sec 33 cycles of 94°C for 45 sec, 56°C for 45 sec and 72°C for 1 min 30 sec and a final extension step at 72°C for 7 min in the thermocycler (MJ research, Waltham, MA, U.S.A.). The resulting PCR product was inserted into the pYES2.1/ V5-His-TOPO vector at the topoisomerase-TA cloning site, which consisted of V5 epitope and multi-histidine coding sequence for fusion expression. *E. coli* DH5α was used for transformation and construction of the vector. Transformants were plated out on LB plates supplemented with 50 μg/mL ampicillin and incubated at 37°C for 1 day. Several colonies were selected by genomic PCR. The PCR was carried out under the same conditions as described above. Plasmids were extracted from positive PCR candidates. DNA sequencing was performed with an ABI 3100 automated sequencer using a BigDye terminator kit (PE Applied Biosystems, Foster City, CA, U.S.A.). Finally, expression vectors of pYESH-FE and pYESL-FE were constructed. The diagram of the expression vectors is shown in Fig. 1.

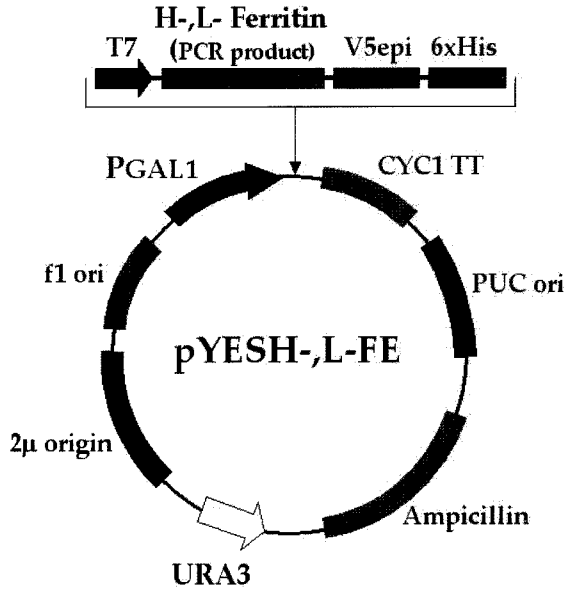

Fig. 1. Construction scheme for expression vectors containing human H- and L-ferritin gene. Amplified human H-ferritin and L-ferritin cDNA were inserted into vectors (pYES2.1/V5-His-TOPO) that contained a topoisomerase-TA cloning site.

Yeast Strain and Transformation

Saccharomyces cerevisiae strain INVS.c1 (His-, Leu-, Trp-, Ura-) was obtained from Invitrogen (Carlsbad, CA, U.S.A.). *S. cerevisiae* was inoculated into YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) at 30°C for 24 h. Turbid cultures were then streaked onto YPD agar plates (YPD plus 1.5% agar). These plates were incubated aerobically at 30°C for 2 days. A single colony was transferred to a flask containing 15 mL of YPD and incubated at 30°C overnight (20 h) with rotary agitation (260 rpm). Cells from the overnight culture were added to a flask containing 50 mL of YPD, adjusted to an optical density of 0.4 at 600 nm and incubated at 30°C with rotary agitation (260 rpm) for 4 h. Cells were collected by centrifugation (4,000 rpm) and resuspended with 2 mL of 1 × LiAc/0.5 × TE solution. Cell suspensions (100 μL) were gently mixed with 1 μL of plasmid (1 μg/μL, pYESH-FE or pYESL-FE), 10 μL of salmon sperm DNA (10 ng/μL), and 700 μL of 1 × LiAc/40 PEG-3350/1 × TE solution. The mixtures were then incubated at 30°C for 30 min and treated with heat-shock at 42°C for 7 min. Cells were pelleted with centrifugation (4,000 rpm) and resuspended with 100 μL of 1 × TE buffer. Cells were finally spread onto uracil deficient SC-U agar plates, which contained 0.67% yeast nitrogen base (without amino acids, with ammonium sulfate; Invitrogen), 2% carbon source (D-glucose or raffinose), 0.01% of adenine, arginine, cysteine, lysine, threonine, tryptophan, uracil, 0.005% of aspartic acid, histidine, isoleucine, methionine, phenylalanine,proline, serine, tyrosine, valine, and 2% agar. These plates were incubated at 30°C for 2 days.

Production of Recombinant Human H- and L-Ferritin

Each H- and L-ferritin transformants were incubated overnight in 15 mL of SC-U medium containing 2% raffinose. The cell pellets were harvested by centrifugation (4,000 rpm), resuspended in 1 mL SC-U medium, then small aliquots were added to a baffled flasks containing 50 mL SC-U medium plus 2% galactose until an optical density of 0.4 at 600 nm was reached. The flasks were then incubated at 30°C shaking (260 rpm) for 12 h to induce recombinant human H- and L-ferritin.

Protein Extraction and SDS-PAGE

To obtain the soluble H- and L-ferritins, cells were extracted using glass beads (Sigma-Aldrich, St. Louis, MO, U.S.A.) and the supernatant was collected by rotor type A1.5S-24 centrifugation at 12,000 rpm (MICRO 17TR, Hanil Science Co., Seoul, Korea). The protein content was determined using a protein assay kit (Bio-Rad, Hercules, CA, U.S.A) based on the Bradford method [2]. Following quantification, 6 μL of 6 × SDS-sample buffer (Sigma-Aldrich) was added to 24 μL of the protein extraction resulting in a total volume of 30 μL and the solution was denatured at 95°C in a heat-block for 5 min. To determine molecular weight, a 20 μL sample was run on a 10% (w/v) SDS-PAGE gel. The denatured proteins were separated using a Mini-protein II cell kit electrophoresis chamber (Bio-Rad). Electrophoresis was conducted at 100 V for 1 h 30 min, and the gel was stained with Coomassie brilliant blue R-250 (Gibco BRL, Gaithersburg, MD, U.S.A.).

Western Blots

For detection of ferritin, proteins were blotted onto a nitrocellulose membrane (Whatman, Dassel, Germany) using a Mini-transfer II kit (Bio-Rad) at 4°C for 16 h. The nitrocellulose membrane was incubated with blocking solution containing 1% (w/v) nonfat milk for 1 h and washed with TBST [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] buffer. Anti-His6 antibody (mouse monoclonal, Roche, Germany) was added as a primary antibody. Consequently, anti-mouse IgG conjugated with horseradish peroxidase was used as a secondary antibody. An ECL kit was used to detect the signal for recombinant H- and L-ferritins (Amersham Pharmacia, Uppsala, Sweden).

Atomic Absorption Spectrometry

For an iron-uptake assay, cells were grown, harvested and resuspended in a SC-U medium supplemented with 0 and 20 mM of ferric citrate. The production of ferritin was induced as described above. After expression, cells were collected by centrifugation (15 min, 5000 × g), washed three times with nano-pure water, and dried at 50°C for 2 days. The dried cells (0.04 g) were digested with 6 mL of concentrated nitric/perchloric acid (2:1, v/v) in volumetric flasks in a flame for 10~15 min. Digested samples were adjusted to a final volume of 100 mL with nano–pure water and impurities removed with a 0.45 μM filter.

The iron quantity was determined by atomic absorption spectrometry AA-6401 (Shimazu, Kyoto, Japan) [7, 10]. The instrument settings were as recommended by the manufacturer. The wavelength was 248.3 nm from a BGC-D2 lamp, with deuterium background correction, and the burner gas mixture was air-acetylene. The iron atomic absorption standard solution used was a commercial product (Accustandard, New Haven, CT, U.S.A.). Working standards were prepared from the stock standard solution by diluting with nitric acid.

Results and Discussion

Construction and Transformation of Human H- and L-Ferritin Gene

In order to study the expression of human H- and L-ferritin in *S. cerevisiae*, the cDNA for human H- and L-ferritin subunits from the previously cloned vectors for the yeast *P. pastoris* [7, 8] were amplified by the PCR and inserted into the expression vector pYES2.1/V5-His-TOPO. More specifically, after conventional PCR, DNA fragments from the coding region for the human H- and L-ferritin genes were subsequently cloned into the topoisomerase-TA cloning site of the pYES2.1/V5-His-TOPO expression vector. After vector constructions were performed, each of the amplified ferritins was observed in a 1.2% agarose gel after electrophoresis. The PCR products resulted in the expected size of H- and L-ferritin cDNA of about 552-bp and 528-bp, respectively. The recombinant expression vectors were finally confirmed by DNA sequencing analysis and named pYESH-FE and pYESL-FE, respectively. The resulting recombinant expression vectors were transformed into the *S. cerevisiae* strain INVS.c1 by LiAc-heat shock method. Several colonies were picked from plates without uracil to identify *S. cerevisiae* containing the plasmid with the selection marker URA3. The PCR using primers specific for each ferritin type was then performed to determine which of these colonies also contained the cloned H- and L-ferritin gene. Five colonies for each subunit were randomly chosen as candidate transformants (Fig. 2). Transformants #2 and #6 were finally selected to express H-ferritin and L-ferritin in *S. cerevisiae*.

Expression of Recombinant Human H- and L-Ferritin in *S. cerevisiae*

For expression of human H-and L-ferritin, transformants

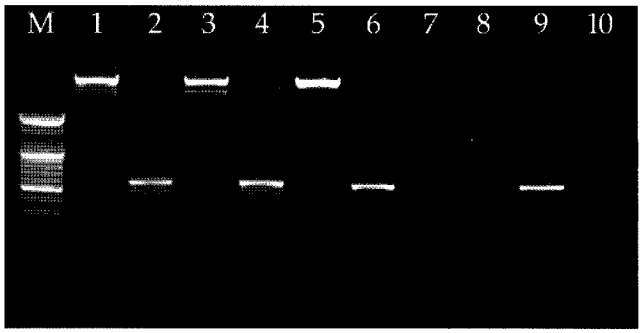

Fig. 2. Agarose gel electrophoresis of PCR product from candidate transformants. Lane M, marker (100 bp DNA ladder); 1-5, H-ferritin transformant; 6-10, L-ferritin transformant. The arrow indicates amplified ferritin PCR products.

were cultured and induced by addition of galactose (2% final concentration) for 12 h. The cell extract samples were resolved in SDS-PAGE and the identity of the expressed ferritin was determined by western blots using Anti-His6 antibody. The expression level of L-ferritin was shown to be less than that of H-ferritin using western blot (Fig. 3B). The expressed H- and L-ferritin each yielded a single band with an estimated molecular mass of about 20 and 19 kDa, respectively (Fig. 3A). This result differed with the reported molecular masses and expressed band pattern for human ferritin subunits in the previous *P. pastoris* reports in which expressed human H- and L-ferritins were about 21~22 and 19~21.5 kDa, respectively. We previously proved that the recombinant human H- and L-ferritins are glycosylated and expressed heterogeneous signal bands in *P. pastoris* [7, 8]. These results were probably due to differences in posttranslational modification between the two different yeast species.

Iron-uptake Capacity of the Recombinant Human H- and L-Ferritin in *S. cerevisiae*

The iron quantities in transformant and control (negative transformant) cells were determined using atomic absorption spectrometry (AAS). Each type of cell was incubated in SC-U medium at 30°C and cells were collected and resuspended in SC-U plus galactose medium supplemented with or without 20 mM ferric citrate. After further cultivation for 12 h, samples were prepared and analyzed (Table 1). The levels of intracellular iron content of H-, L-ferritin, and negative transformant increased slightly from 1.2 fold to 1.6 fold in the media without ferric citrate. With 20 mM ferric citrate supplementation, the iron uptake of H-, L-ferritin, and negative transformant was 9.64, 5.32, and 5.26-fold more than that of negative transformant with 0 mM ferric citrate. The iron content in the L-ferritin and negative transformant was not significantly different with 20 mM ferritic citrate.

The greatest amount of intracellular iron was observed in the H-ferritin transformant (Table 1). These results are due to the ferroxidase site of human H-ferritin as previously

Table 1. Atomic absorption spectrometry of intracellular iron content.

Iron Concentration in Medium	Samples		
	Negative transformant	L-ferritin transformant	H-ferritin transformant
Fe 0 mM	101±26[a]	128±35	163±23
Fe 20 mM	532±45	538±67	974±36

a : Unit (μg/g)

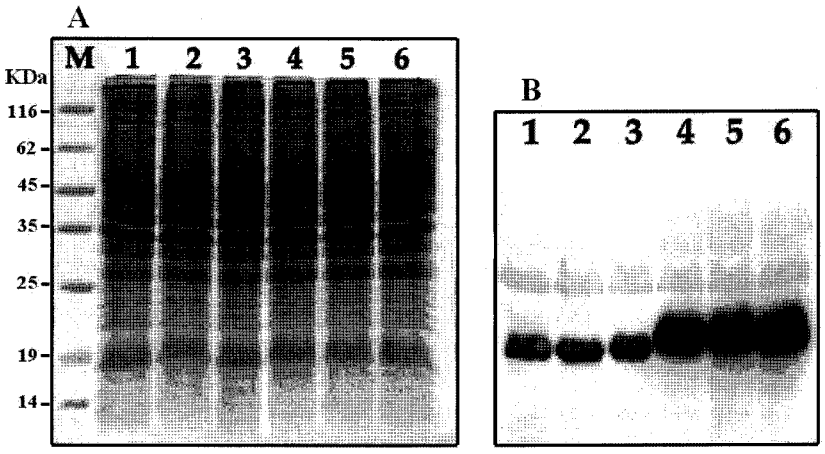

Fig. 3. Expression of recombinant H- and L-ferritins in *S. cerevisiae*. Samples were loaded onto a 10 % SDS-PAGE. (**A**) Proteins were stained with Coomassie brilliant blue. (**B**) Western blot analysis. Lane M, protein standard marker; 1-3, cell extracts from L-ferritin transformant; 4-6, H-ferritins transformant. The arrow indicates expressed ferritins.

reported [11, 12, 14, 21]. It is suggested that recombinant human H-ferritin has a major role in iron accumulation and oxidation. The measured amounts of iron content in H-ferritin transformant were 974 ± 36 μg/g dry cell. The iron content in the H-ferritin transformant in this study was 3.1 fold less than that in the H-ferritin transformant of *P. pastoris* [7], but 1.9 fold higher than in the tadpole- and human H-ferritin transformant of *S. cerevisiae* reported previously [6, 19]. However, *S. cerevisiae* is generally recognized as a safe (GRAS) organism and would likely be more widely used in industrial applications than *P. pastoris*. The vector containing topoisomerase-TA site was able to insert the ferritin gene rapidly into this cloning site. Our results showed that recombinant H-ferritin in *S. cerevisiae* has a functional property of iron storage *in vivo*. The H-ferritin transformant in this study could have a useful application in iron-fortification of functional foods. Such a strategy using ferritin transformants is being extended to *in vivo* study of the efficiency of iron supplementation for animal feed.

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초록국문

*Saccharomyces cerevisiae*을 이용한 사람의 H-, L-ferritins의 발현 연구

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효모에서 사람의 H-, L-ferritin을 생산하기 위해서, 기존에 복제된 vector를 사용하였으며, 단백질을 발현하기 위해서 각각의 증폭된 ferritin 유전자를 GAL1 promoter에 의해 조절되는 pYES2.1/V5-His-TOPO 효모 발현 vector에 삽입하였다. Western blot 분석을 통해서 사람의 H-, L-ferritin subunits을 함유한 재조합 효모에서 사람의 ferritin이 발현된 것을 확인할 수 있었다. 또한 Atomic absorption spectrometry(AAS) 분석을 통해서 형질변환된 효모의 철 함유량이 대조군과 비교하여 1.6~1.8배 증가한 것을 확인하였다. 향후ferritin이 함유된 형질변환 효모를 사용하여 잠재적으로 철이 강화된 영양성분을 기능성 식품과 사료에 이용할 수 있을 것이다.