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Purification and Glycosylation Pattern of Human L-Ferritin in *Pichia pastoris*

LEE, JUNG-LIM, SEUNG-NAM YANG, CHEON-SEOK PARK, DOOIL JEOUNG[1], AND HAE-YEONG KIM*

College of Life Sciences, Kyung Hee University, Suwon 449-701, Korea
[1]Department of Microbiology, College of Natural Sciences, Kangwon National University, Chuncheon 200-701, Korea

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Abstract Ferritin is an iron storage protein found in most living organisms. For expression and industrial use, human light chain ferritin (L-ferritin) was cloned from human liver cDNA library and expressed in *Pichia pastoris* strain GS115. The recombinant L-ferritin in *Pichia pastoris* was glycosylated. In a fed-batch culture, the cell mass reached about 57 g/l of dry cell weight, and the L-ferritin in the cell was increased to about 95 mg/l after 150 h. In an atomic absorption spectrometry analysis, the intracellular content of iron in the L-ferritin transformant was measured as 1,694±85 μg/g, which is 5.4-fold more than that of the control strain. This L-ferritin transformant could serve as iron-fortified nutrients in animal feed stock.

Key words: Fed batch, human L-ferritin, iron, *Pichia pastoris*

Ferritin is the major iron-storage protein found in most cell types of humans and other vertebrates [11, 21]. It plays a central role in protecting the cell from damage due to iron-catalyzed production of reactive oxygen species [1], and keeping iron in a metabolically accessible form [17]. The large sphere constituting a ferritin particle consists of an outer protein coat composed of 24 subunits and an inner core composed of up to 4500 ferric ions in the form of a crystalline hydroxy phosphate mineral. Vertebrate ferritins contain two types of subunits, termed light (L) and heavy (H) chains, depending upon the tissue of origin.

Even though H and L chains of ferritins share 55% homology in human, they are different in size, surface charge, immunological reactivity, and presumably iron-loading properties [3, 9]. The L-ferritin promotes a higher growth of Fe (II) clusters than the H-ferritin, and L-ferritin is more resistant to physical denaturation than H-type ferritin [18, 19]. In both prokaryotic and eukaryotic expression systems, the production of recombinant ferritin has been investigated for biochemical studies [19, 20], however, in *Pichia pastoris*, the expression and characterization of recombinant L-ferritin has not been studied for its potential industrial use. Among yeast expression systems, *Pichia pastoris* especially uses the powerful AOX1 promoter to produce higher levels of recombinant protein, and the cloned gene is integrated into the genomic DNA. This cloned gene is genetically stable, because *Pichia pastoris* is its host system and the glycosylated protein structures are more similar to human proteins than to the hyperglycosylated proteins in *S. cerevisiae*. This gene also creates easily a high cell density culture and performs the scale-up process [6, 7, 15].

For the production of human L-ferritin and the development of iron-fortified nutrient food and feed, L-ferritin cDNA from the human liver cDNA library was cloned and than expressed in *Pichia pastoris*. The result obtained on the expression and glycosylation of human L-ferritin from *Pichia pastoris* is described herein.

Material and Methods

Cloning and Transformation

Pichia pastoris strains GS115 (Mut$^+$) and expression vector pPICZαA were obtained from Invitrogen (San Diego, U.S.A.). The pPICZαA plasmid contains a zeocin selectable marker, an alcohol oxidase 1 (AOX1) promoter-terminator cassette and a multicloning site. Human L-ferritin cDNA was amplified by PCR using the liver cDNA library (Clontech, Palo Alto, U.S.A.). The primers for cloning were based on the human L-ferritin sequence from GenBank (Accession no. M11147) and were commercially synthesized (Bioneer, Taejon Korea). The PCR was carried out as follows; a first denaturation step at 94°C for 5 min, 30 cycles at 94°C for 1 min, at 49°C for 1 min, at 73°C for

*Corresponding author
Phone: 82-31-201-2660; Fax: 82-31-204-8116;
E-mail: hykim@khu.ac.kr

1 min, and a final extension step at 73°C for 5 min in a thermocycler (MJ research, Waltham, U.S.A.). This PCR product was cloned in frame into the pPICZαA vector at the 5' *Eco*RI and 3' *Xba*I sites. The constructed vector was linearized with *Pme*I and transformed into GS115 by electroporation using a gene pulser set (Bio-Rad, Hercules, U.S.A.) at 1,500 V, 25 F, and 200 Ω, using a 0.2-cm cuvette [4]. Transformants were plated on YPDS (yeast extracts, peptone, dextrose, and sorbitol) plates and incubated at 30°C for 3 days. Several integrated colonies were selected by genomic PCR, and PCR was carried out under the same conditions as described above.

Media Composition

The buffered minimal glycerol medium (BMGY) contained biotin 0.4 mg, yeast nitrogen base without amino acids 13.4 g, and glycerol 10 g in l-l medium. Also, the minimal glycerol medium was supplemented with 100 mM potassium phosphate, pH 6.0. The buffered minimal methanol medium (BMMY) was identical to the minimal glycerol medium but contained 0.5% (v/v) methanol instead of 1% (v/v) glycerol. The fermentation basal salts medium contained 85% (w/v) H_3PO_4 26.7 ml, $CaSO_4$ 0.93 g, K_2SO_4 18.2 g, $MgSO_4 \cdot 7H_20$ 14.9 g, KOH 4.13 g, glycerol 40 g, ferric citrate 4.89 g, casamino acid 10 g, and 4.3 ml trace elements in 1-l medium.

Flask and Batch Culture for Recombinant L-Ferritin in *Pichia pastoris*

Transformants were incubated overnight in a buffered minimal glycerol medium, and harvested, and then the collected cell pellets were resuspended in a buffered minimal methanol medium. Cells were cultured in baffled shaking flasks or fermenter for 5 days at 30°C. Methanol was added to make a final concentration of 0.5% (v/v) methanol every day [5]. Zeocin with a concentration of 50 μg/ml was added to the medium (Invitrogen, San Diego, U.S.A.). To determine glycosylation of the protein, 40 mg/l tunicamycin was added to the BMMY medium. Then, cells were grown as described above.

SDS-PAGE and Western Blots

For the determination of the molecular weight, cell extracts were run on a 13% (w/v) SDS-PAGE gel and stained with Coomassie brilliant blue R [10, 12]. For the detection of ferritin, proteins were blotted onto nitrocellulose membrane (Schleicher and Schuell, Keen, U.S.A.). The nitrocellulose membrane was incubated with blocking solution containing 1% (w/v) nonfat milk for 1 h and washed with TBST buffer. The L-ferritin specific monoclonal antibody was added as a primary antibody, and antimouse IgG conjugated with horseradish peroxidase was used as a secondary antibody. An ECL kit was used to detect the signal specific for L-ferritin (Amersham Pharmacia, Uppsala, Sweden).

Purification of Recombinant L-Ferritin

To purify the soluble L-ferritin, cells were extracted using glass beads (Sigma, U.S.A.), and the supernatant was collected by centrifugation. The cell extract was heated at 72°C for 10 min, and the supernatant was precipitated with 80% (w/v) saturated ammonium sulfate. After dialysis in 20 mM Tris/HCl (pH 7.4) and 50 mM NaCl, the sample was loaded onto a Superose 12 column [13]. Using the FPLC system (Pharmacia, Uppsala, Sweden), the column was eluted with 100 mM NaCl in 20 mM Tris/HCl (pH 7.4). The fraction containing ferritin was collected and concentrated with microcon 10 (Millipore, Billerica, U.S.A.).

Fermentation

Selected transformant was inoculated in a shake flask containing 100 ml of BMGY medium. After saturation, the culture was transferred to a fermenter containing 1-l of basal salts medium with an antifoam agent and 1% (w/v) casamino acid. The fermenter was run at 30°C and 550 rpm. An automatic regulator maintained the pH, agitation, and aeration. After batch culture fermentation, glycerol was fed into the fermenter at the rate of 1 g/l/h. When the cell dry weight reached approximately 30 g/l, the feeding of methanol was started at the rate of 1 ml/l/h until the fermentation was stopped after 100 h. Other supplements of trace elements were supplied by initiating methanol feed containing 12 ml/l of trace elements. Casamino acid had been added to 1% (w/v) of the initial fermenter volume. Samples of the transformant culture were taken daily for analysis. Sample extracts were resolved by SDS-PAGE and confirmed by Western blotting. Finally, the expression ratios of L-ferritin were determined by image scanning densitometry (Bioneer, Taejon, Korea).

Atomic Absorption Spectrometry

For an iron-uptake assay, cells were grown, harvested, and resuspended in a buffered minimal methanol medium supplemented with 20 mM ferric citrate. The production of ferritin was induced as described above. After induction, cells were collected by centrifugation (7 min, 4,000 ×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 in a flask with nano-pure water reached a final volume of 100 ml. The unknown quantity of iron was determined by atomic absorption spectrometry AA-6401 (Shimazu, Kyoto, Japan). The instrument settings were made according to the standard procedure recommended

by the manufacturer. The wavelength was 248.3 nm with a 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, U.S.A.). Working standards were prepared from the stock standard solution by diluting with dilute nitric acid solution.

RESULTS AND DISCUSSION

Construction and Transformation of Human L-Ferritin cDNA

To study the expression of human L-ferritin in *Pichia pastoris*, the expression vector pPICZαA was used to increase the expression and solubility of ferritin in the cell [22]. For obtaining the human L-ferritin gene, the cDNA from the human liver cDNA library was amplified by PCR. After PCR was performed, *Eco*RI and *Xba*I DNA fragments of the coding region for the human L-ferritin gene were digested and subsequently cloned into the multiple cloning sites of the pPICZαA vector. The resulting recombinant expression vector was named as pPICL-FE (Fig. 1). pPICL-FE was linearized with *Pme*I and transformed into the *Pichia pastoris* GS115 strain by electroporation. To aid selection of the L-ferritin transformant, several colonies from plates containing zeocin as a selection marker were chosen, and PCR was performed to verify the integration of L-ferritin cDNA into the *Pichia pastoris* GS115 chromosome.

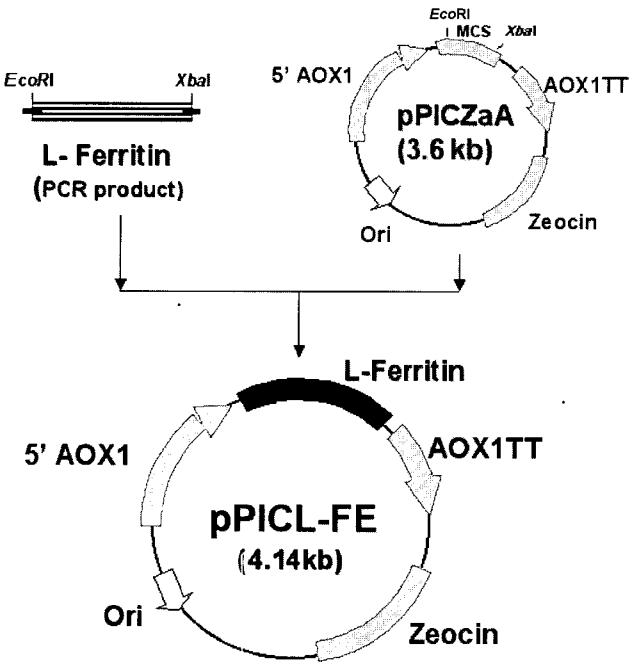

Fig. 1. Construction scheme of the expression vector containing the human L-ferritin gene.
The amplified human L-ferritin cDNA regions were inserted into vectors that contained unique *Eco*RI and *Xba*I restriction enzyme sites.

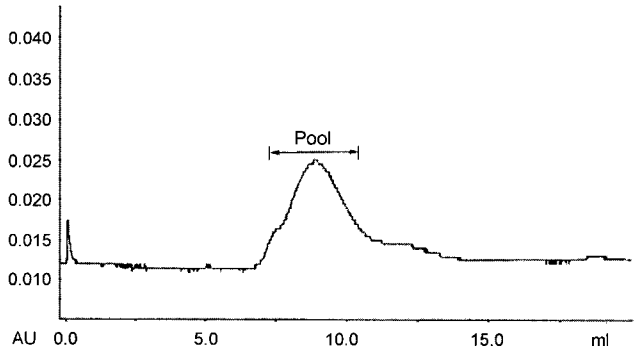

Fig. 2. Chromatogram of recombinant L-ferritin.
The recombinant L-ferritin was loaded onto a Superose 12 column. The column was eluted with 100 mM NaCl in 20 mM Tris/HCl (pH 7.4). The flow rate and the fraction volume were 0.5 ml/min and 1 ml, respectively. The arrow indicates pool of ferritin fraction.

Purification and Glycosylation of Recombinant L-Ferritin in *P. pastoris*

For the expression of human L-ferritin, the transformant was cultured and induced by the addition of methanol (0.5% final concentration) for 5 days. A sample was taken from the fermenter on the final day. The purification of L-ferritin was performed at room temperature, because ferritin is a thermostable protein [8, 19]. Soluble protein was heat-treated to eliminate cellular protein from the transformant. The sample was loaded onto a gel filtration column by the FPLC system, and the chromatogram of this sample is shown in Fig. 2. The fraction containing L-ferritin was collected and concentrated with microcon 10. The purified L-ferritin was resolved by SDS-PAGE and determined by Western blotting analysis. The result showed 2 bands, estimated to have a molecular mass of 19 and 21 kDa (Fig. 3). Owing to this unexpected phenomenon, it was supposed that these heterogeneous signal bands were due to differences in the post-translational modification of L-ferritin. It has been reported that the recombinant tadpole ferritin was glycosylated in *Saccharomyces cerevisiae* [20]. To confirm whether the recombinant L-ferritin was glycosylated in cytoplasm, tunicamycin was added to the induction media [2]. As shown in Fig. 4, the heterogeneity of the recombinant L-ferritin was changed into a single band of 19 kDa after treatment with tunicamycin. The human ferritin has been found to be glycosylated at two sites in the amino acid sequence and is known to go through various post-translational modifications in the cell [16, 21]. As tunicamycin prevents the first synthetic step of the branched oligosaccharide, N-linked glycosylation of Asn-X-Ser/Thr residues of L-ferritin did not occur in *Pichia pastoris* [14]. Therefore, the result led us to conclude that

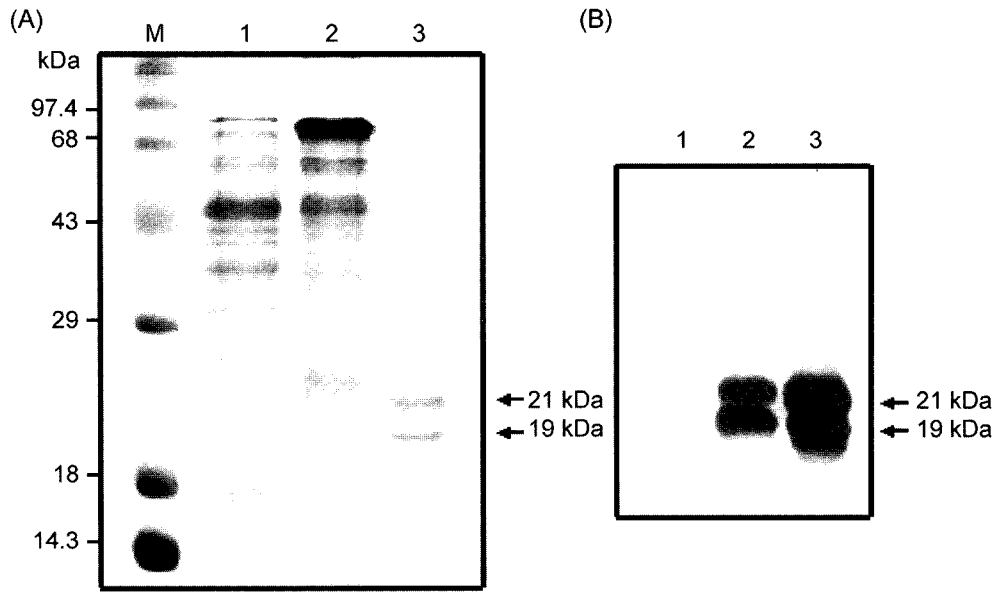

Fig. 3. SDS-PAGE pattern of purified L-ferritin.
The purified L-ferritin was loaded onto 12% SDS-PAGE. (A) The proteins were stained with Coomassie brilliant blue and (B) Western blot analysis. M: protein standard marker; lane 1: the cell extract of control strain; lane 2: the cell extract of transformant; lane 3: the purified L-ferritin. The arrows indicate ferritins.

glycosylation of L-ferritin was one of diverse aspects of expression in *P. pastoris.*

Fermentation for Recombinant L-Ferritin in *Pichia pastoris*

Fed-batch fermentation was employed to achieve a high cell density culture for the high level production of L-ferritin in *Pichia pastoris.* At the beginning of the fed-batch fermentation, a carbon source and nutrients for the culture of cells were added periodically to the fermenter. A sufficiently high cell mass was achieved during the growth phase by supplying glycerol as the carbon source. Additional glycerol was fed in after the carbon source was exhausted. After 50 h of fermentation, the induction phase

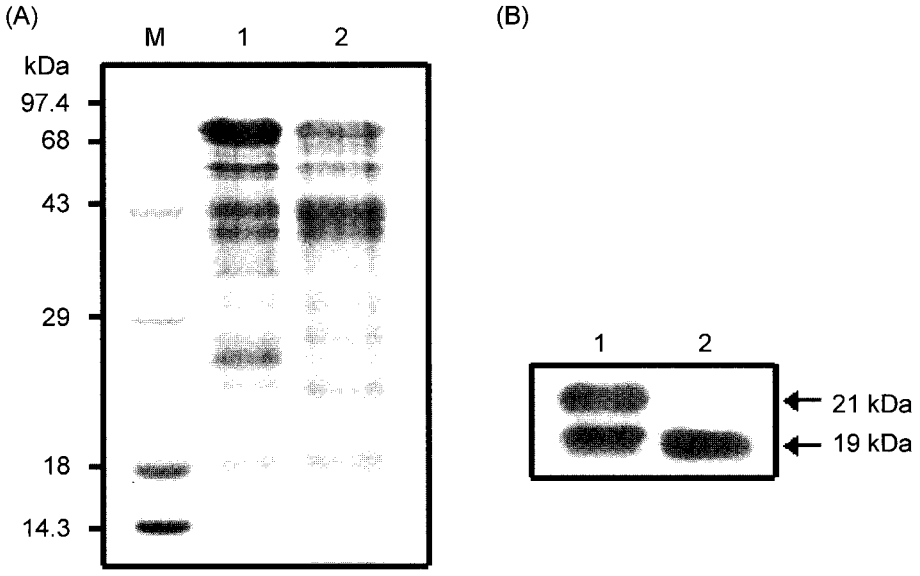

Fig. 4. Characterization of expressed L-ferritin with/without tunicamycin.
Tunicamycin was added to induction media. The cell extract were loaded onto 13% SDS-PAGE, for Western blot analysis. (A) The proteins were stained with Coomassie blue. (B) The proteins were detected with Western blot analysis. M: protein standard marker; lane 1: the sample was treated without tunicamycin; lane 2: the sample was treated with tunicamycin. The arrows indicate ferritins.

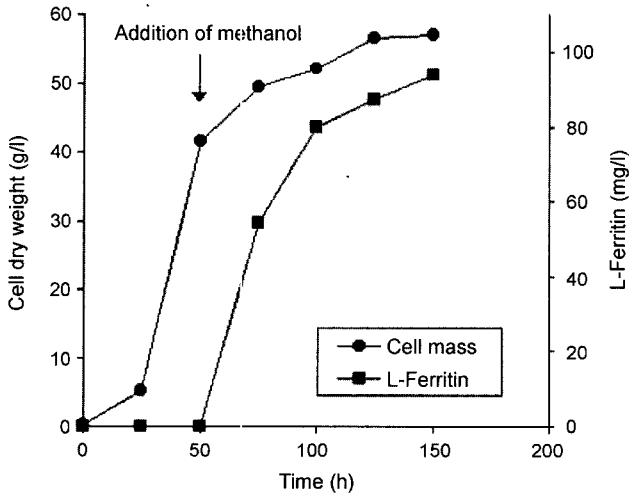

Fig. 5. Expression of L-ferritin and growth of transformant in a fermenter.

The culture was grown in glycerol fed-batch for 50 h. L-Ferritin expression was induced by methanol fed-batch for 100 h. Samples of the transformant culture were taken daily for analysis.

was started using a methanol feed as carbon source. The trace salts and nitrogen source supplementations were also started at the time of induction. The expression of L-ferritin was followed by the analysis of samples removed every 24 h from the fermenter broth. While cell extracts were analyzed by SDS-PAGE and Western blotting, the quantity of expressed ferritin was measured by densitometry. Although the cell mass had already reached a value of 41.5 g/l of cell dry weight, the L-ferritin was induced only after methanol feeding started. The expression of L-ferritin in the cell was increased to 95 mg/l of cell dry weight at 150 h of fermentation by feeding with methanol (Fig. 5). The yield of cell mass achieved in the fed-batch fermentation was 12 times higher than the yield of the batch culture fermentation (data not shown).

Iron-Uptake Capacity of the Recombinant Human L-Ferritin in Cell

The iron contents of the transformant and control cells were determined using atomic absorption spectrometry (AAS). Each cell was incubated in a BMMY/zeocin medium at 30°C. The saturated culture was collected and resuspended in the medium that was supplemented with and without 20 mM ferric citrate. After further cultivation for 150 h, samples were prepared and analyzed (Table 1). The level of iron content drastically increased in the transformant, while that of the control strain slightly increased. The measured amounts of iron content in the transformant and the control were 1,694±85 and 314±58 μg in 1 g of dry cells, respectively. The iron content in this L-ferritin transformant was about 5.4-fold more than that in the control cells. This result was due to the higher growth of

Table 1. Atomic absorption spectrometry analysis of iron content in L-ferritin transformant.

(μg/g)[a]

Iron concentration[b]	Control[c]	Transformant[c]
Iron (20 mM)	314±58	1,694±85
Iron (0 mM)	175±77	248±68

[a]Represents iron weight per 1 g dry cell weight.

[b]Iron concentration in media.

[c]Values were obtained from at least three repeated experiments.

Fe clusters in human L-ferritin, as previous reported [19]. The above results show that recombinant L-ferritin in *Pichia pastoris* has the functional property of storing iron *in vivo*. Therefore, this *P. pastoris* which contains L-ferritin could serve as an iron-fortified nutrient in food and feed.

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