Process Biochemistry 47 (2012) 2300–2305

Contents lists available at SciVerse ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Overexpression of *HAC1* gene increased levels of both intracellular and secreted human kringle fragment in *Saccharomyces cerevisiae*

Tae-Hee Lee [a], Yi-Hyun Bae [a], Myoung-Dong Kim [b], Jin-Ho Seo [a,*]

[a] Department of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Republic of Korea
[b] School of Bioscience and Biotechnology and Institute of Bioscience and Biotechnology, Kangwon National University, Chuncheon 200-701, Republic of Korea

ARTICLE INFO

Article history:
Received 23 May 2012
Received in revised form 17 August 2012
Accepted 7 September 2012
Available online 14 September 2012

Keywords:
HAC1
IRE1
Saccharomyces cerevisiae
Unfolded protein response
LK8

ABSTRACT

Effects of overexpression of an unfolded protein response (UPR) gene were investigated in recombinant *Saccharomyces cerevisiae* strains harboring 16 copies of the gene for human kringle protein, LK8. In *S. cerevisiae*, Hac1p plays a crucial role as a transcription activator and Ire1p as endonuclease acting on the *HAC1* transcript to remove an intron and induce the UPR. The disruption of the two genes was detrimental to LK8 expression, and interestingly, the *hac1Δ* strain was not able to utilize galactose as a carbon source and concomitantly delayed cell growth compared with the wild type and the *ire1Δ* strains. In a complementation test, the growth defect was partially recovered by the overexpression of the *HAC1* gene controlled by the *GAL1* promoter. Additional activation of UPR was mediated by the *GAL1* promoter driven coexpression of the *HAC1* gene and enhanced the intracellular and secreted levels of LK8 by 4- and 1.6-fold, respectively. The UPR was essentially required for the heterologous production of LK8. Furthermore, Hac1p is the factor promoting LK8 protein production as well as cell growth in recombinant *S. cerevisiae* strains. This result indicates that the additional activation of UPR might be a good option for overproduction of heterologous proteins in *S. cerevisiae*.

1. Introduction

Production of heterologous proteins in eukaryotic hosts has advantages due to the ability for folding and secretion of target proteins though their secretory machinery [1,2]. Many organisms such as *Pichia pastoris* [3], *Kluyveromyces lactis* [4,5], *Hansenula polymorpha* [6] and *Saccharomyces cerevisiae* [7] have been used as hosts. Among them, *S. cerevisiae* is one of the most popular workhorses owing to good exploitation to food and pharmaceutical industries [8]. Genomic databases, tools and methods for genetic manipulations of the baker's yeast *S. cerevisiae* have been assorted as well. However, this yeast inherently exhibits a protein expression level lower than the prokaryotic hosts. To solve this problem, a method for genomic integration of multiple copies of the target gene has been developed using a transposable element [9], which enabled the high-level expression of the *Escherichia coli lacZ* gene in *S. cerevisiae*. On the other hand, it was frequently observed that the recombinant *S. cerevisiae* strain retained most of the expressed proteins in the endoplasmic reticulum (ER), whereby the yeast only secreted a slightly increased amount of the target protein [10,11]. Overproduction of a heterologous protein led to the accumulation of an improperly folded protein, resulting in ER-overload [12]. Such accumulation often induces a stress response pathway, the unfolded protein response (UPR) in host cells. This response is conserved in all eukaryotic organisms [13]. To date, it is known that activation of UPR induces expression of various genes such as ER-localized chaperones, foldases, proteases for ER-associated degradation, and proteins for vesicle formation [14–16]. As secretion of heterologous proteins in eukaryotes is originated from ER, UPR has been believed to be a major control unit in protein secretion and homeostasis.

UPR activates expression of three components in *S. cerevisiae*: Ire1p, Rlg1p and Hac1p. Ire1p is an ER transmembrane protein with its N-terminal domain, which contains an ER targeting sequence, and senses unfolded proteins [14,17,18]. The C-terminal half of Ire1p contains both protein kinase and site-specific endonuclease activities [17,19]. When Ire1p dimerizes, it is autophosphorylated *in trans*, followed by activation of its endonuclease activity that acts on the *HAC1* transcript to remove the 252 nucleotide intron located within the predicted stem-loop structure [14,20]. Activity of Rlg1p, a tRNA ligase, is required to ligate the transcript cleaved by Ire1p. The spliced *HAC1* mRNA is then translated upon the removal of the intron. Hac1p is a transcription factor that activates the unfolded protein response (UPR) pathway [14,21]. As described, Ire1p, Rlg1p and Hac1p are the key factors for the induction of the UPR genes in yeast since Hac1p is synthesized only after the activation of Ire1p and Rlg1p in response to ER stress.

* Corresponding author. Tel.: +82 2 880 4855; fax: +82 2 873 5095.
E-mail address: jhseo94@snu.ac.kr (J.-H. Seo).

1359-5113/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.procbio.2012.09.006

In an effort to improve the secretion of heterologous proteins, Hac1p was coexpressed in recombinant *S. cerevisiae* strains [22]. The constitutive expression increased secretion of *Bacillus amyloliquefaciens* α-amylase [22] by a 1.7-fold and surface expression of *Burkholderia gladioli* esterase [23] by a 1.25-fold. However, continuous expression of the *HAC1* gene did not increase the production of *Trichoderma reesei* endoglucanase [22]. Based on the literature reported elsewhere, an influence on the target protein expression was discussed only about the secreted parts. Level changes of the target protein trapped inside seem to be critical since the results reflect total expression changes and functions of UPR in *S. cerevisiae* strains producing heterologous proteins. Thus, this work was focused on analysis of changes in both intra- and extracellular protein concentrations in response to the additional expression or inactivation of the *IRE1* and *HAC1* genes in recombinant *S. cerevisiae* strains. A production system of the kringle fragment of human apolipoprotein(a) [24], termed as LK8, which has strong anti-angiogenesis activity [25], was used as a model protein. LK8 is a three disulfide-linked 86 amino-acid polypeptide, which inhibits endothelial cell migration and suppresses formation of new capillaries.

This work provides experimental evidence to prove that an *IRE1*-dependent activation of UPR is essential and the coexpression of Hac1p exerts a profound influence on improving the expression level of LK8.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli DH5 was used for plasmid preparation. A recombinant *S. cerevisiae* 2805/MδLK8 (*MATα pep4::HIS3 prb1 can1 his3 ura3-52*) strain harboring 16 copies of the LK8 expression cassette in the genome [24] was used. The LK8 expression cassette was comprised of the *GAL1* promoter, α-factor signal sequence, LK8 structural gene and *CYC1* terminator.

Plasmids pWAL100 and pWBR100 were used as mother vectors for construction of knockout alleles [26]. Disruption vectors pWALhac1, pWBRhac1, pWALire1, and pWBRire1 were constructed and generated as complementary knockout cassettes for disruption of the *HAC1* and *IRE1* genes. The constructed null mutants were confirmed by diagnostic PCR. Phenotypic assay for inositol auxotrophy and sensitivity to tunicamycin was validated for the *HAC1* and *IRE1*-disrupted strains. Two versions of the *S. cerevisiae HAC1* gene were cloned in p426GAL1 (ATCC 87833), an expression vector containing the *GAL1* promoter. The $HAC1^{i}$ gene coding for intronless 238-amino acids was generated by overlapping PCR with appropriate primers and ligated to plasmid p426GAL1 at the *Bam*HI and *Cla*I loci. The constructed plasmid pTL305 consists of the spliced (induced) sequence of the intronless *HAC1* gene ($HAC1^{i}$) coding for an induced form of 238-amino acid protein ($Hac1p^{i}$). The unspliced (uninduced) *HAC1* gene of which the transcript encodes 230-amino acid protein ($Hac1p^{u}$) was obtained by PCR. Plasmid pTL307 comprising the *GAL1* promoter for expressions of *S. cerevisiae* $HAC1^{u}$ was also constructed based on the same method for plasmid pTL305. Primers used in this study were listed in Table 1. Plasmids p426GAL1, pTL305 and pTL307 were digested by *Stu*I. The linearized DNA fragments were transformed to a recombinant *S. cerevisiae* 2805/MδLK8 strain. An empty vector p426GAL1 was used as control for transformation. Plasmids pTL305 and pTL307 were used for single-copy coexpression of the $HAC1^{i}$ and the $HAC1^{u}$ genes. The transformants were confirmed by colony PCR with appropriate primers. Recombinant *S. cerevisiae* strains used in this study were listed in Table 2.

2.2. Media and culture conditions

LB medium (1% NaCl, 1% tryptone, and 0.5% yeast extract) was used for *E. coli* cultivation. SC (synthetic complete) plates without uracil [27] were used for selection of the transformants harboring the *URA3* or the *Kl URA3* genes. A medium containing 5-fluoorotic acid was used for counterselection [27]. YEPG (1% yeast extract, 2% peptone and 2% galactose) plates were used for complementation experiments. A bench-top fermentor (Bioengineering AG, Wald, Switzerland) was used for batch cultivations with a 1.5-l working volume of medium in a 3-l jar. Flask cultures and batch fermentations were performed in YEPDG medium (1% yeast extract, 2% peptone, 2% glucose and 2% galactose). Inoculums were adjusted to OD_{600} = 0.5. An agitation speed of 500 rpm and an aeration of 1 vvm were maintained throughout the cultivation. Medium pH was adjusted at 5.5 with 2 N HCl or 2 N NaOH, and the temperature was maintained at 30 °C. All the values were averaged from the duplicate batch fermentations.

2.3. Analysis

Dry cell mass concentration was measured with a spectrophotometer at 600 nm. Concentrations of glucose and galactose were determined using the glucose kit (Young-Dong Pharm., Seoul, Korea) and the 3,5-dinitrosalicylic acid (DNS) method, respectively. Ethanol concentration was measured by a gas chromatograph (Younglin, Anyang, Korea). Intracellular proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblot was performed using the anti-LK8 rabbit polyclonal antibody (lab stock). Samples for intracellular LK8 and Kar2p were prepared by adjusting the cell density in TBS buffer ($10\,OD_{600}\,ml^{-1}$) and by following boiling and breaking the cells with glass beads (Sigma Chemical Co., St. Louis, MO, USA). To prevent the plateau effect in immunoblots, all samples were serially diluted and loaded for SDS-PAGE. Samples for quantitative detection of the secreted LK8 were prepared directly from the supernatant of culture media at 24 h and 48 h of each strain. Concentrations of the secreted LK8 protein were also measured by an enzyme-linked immunosorbent assay (ELISA) based on linearity of the predetermined standard curve with the *r*-squared value of 0.9896. Peroxidase conjugated anti-rabbit IgG (Sigma Chemical Co.), LK8 polyclonal antibody, and 3,3′,5,5′-tetramethylbenzidine (TMB) dihydrochloride (Sigma Chemical Co.) as a peroxidase substrate were used. Absorbance was measured at 450 nm using a VERSAmax microplate reader (Molecular Device, Sunnyvale, CA, USA). The purified LK8 protein was used as a standard. All data were obtained from at least two or more independent experiments and averaged.

3. Results

3.1. Production of LK8 in hac1Δ and ire1Δ strains

To analyze the effect of UPR on LK8 production, the *HAC1* and *IRE1* genes were eliminated since both genes are known to be required for the induced activation of UPR. YTL59 (*hac1Δ*) and YTL60 (*ire1Δ*) strains were constructed from *S. cerevisiae* 2805/MδLK8 (Table 2) and batch fermentations were performed in YEPDG media. For 24 h and 48 h of cultivations, the levels of LK8 secreted in the YTL59 and YTL60 strains were reduced to a range of 36–50% compared with the control YTL30 strain (Fig. 1A).

Table 1
Primers used in this study.

Name	Sequence	Relevant work
F_AL_hac1_BamHI	CGGGATCCTACAATGAAAAACAACACCAAGCTGC	pWALhac1
R_AL_hac1_NcoI	CATGCCATGGAGTGGCGGTTGTTGTCGTAGGTG	″
F_BR_hac1_BamHI	CGGGATCCAGACAATCGCAAGAGGGTATAATTTTT	pWBRhac1
R_BR_hac1_NcoI	CATGCCATGGCTCATTGGCCCGGAGTTAGGGG	″
F_AL_ire1_BamHI	CGGGATCCGAAGTAATGAACTTAAATGCTATTATACA	pWALire1
R_AL_ire1_NcoI	CATGCCATGGTTTTCAAAGTGCTAAAATATTAATTCCTC	″
F_BR_ire1_BamHI	CGGGATCCCATGTTCATGCCCCTCTGCTTCTT	pWBRire1
R_BR_ire1_NcoI	CATGCCATGGTCTCAGGAACAAAAAGGAATATCTTTG	″
F_hac220	ACTATGGAAATGACTGATTTTGAACTAA	$HAC1^{i}$ template generation
R_hac220	CTGGATTACGCCAATTGTCAAGATC	
F_hac1_BamHI	CGGGATCCACTATGGAAATGACTGATTTTGAACTAA	$HAC1^{i}$, $HAC1^{u}$
R3_hac238_ClaI	CCATCGATTCATGAAGTGATGAAGAAATCATTCAATTCAAATGAATT-CAAACCTGACTGCGCTTCTGGATTACGCCAATTGTCAAGATCA	$HAC1^{i}$
R_hac238_OP	TCTGGATTACGCCAATTGTCAAGATC	$HAC1^{i}$
R_hac230_ClaI	CCATCGATTCACTGTAGTTTCCTGGTCATCGTAA	$HAC1^{u}$

Table 2
Recombinant *S. cerevisiae* strains used in this study.

Strain	Genotype	Used plasmid	Reference
2805	*MATα prb1 can1 his3 ura3-52 pep4::HIS3*		Cha et al. [24]
2805/MδLK8	2805 *Ty-δ*::P_{GAL1}-*MFα*-LK8-T_{cyc1}-neo^r	MδLK8	Cha et al. [24]
YTL30	2805/MδLK8 *ura3*::*URA3*-P_{GAL1}-T_{cyc1}	p426GAL1	This study
YTL31	2805/MδLK8 *ura3*::*URA3*-P_{GAL1}-*HAC1*^{i}-T_{cyc1}	pTL305	This study
YTL32	2805/MδLK8 *ura3*::*URA3*-P_{GAL1}-*HAC1*^{u}-T_{cyc1}	pTL307	This study
YTL59	2805/MδLK8 *hac1Δ ura3*::*URA3*-P_{GAL1}-T_{cyc1}	p426GAL1	This study
YTL60	2805/MδLK8 *ire1Δ ura3*::*URA3*-P_{GAL1}-T_{cyc1}	p426GAL1	This study
YTL63	2805/MδLK8 *hac1Δ ura3*:: *URA3*-P_{GAL1}-*HAC1*^{i}-T_{cyc1}	pTL305	This study
YTL64	2805/MδLK8 *hac1Δ ura3*:: *URA3*-P_{GAL1}-*HAC1*^{u}-T_{cyc1}	pTL307	This study

Based on the secreted LK8 for the YTL59 and YTL60 strains, the LK8 content was significantly decreased by disruption of the *HAC1* and *IRE1* genes. Furthermore, no LK8 protein was detected in the *hac1Δ* mutant through an immunoblot analysis (Fig. 1B). A decrease in secreted and intracellular levels of LK8 in both strains suggested that the *HAC1* and *IRE1* genes were equally important in LK8 expression.

3.2. Patterns of galactose consumption in hac1Δ and ire1Δ strain

Elimination of the *HAC1* gene halted cell growth and galactose assimilation during the LK8 induction phase (Fig. 2A and B). The galactose uptake rate was close to zero after the depletion of glucose for the *hac1Δ* strain, in which the UPR induction could not occur. In addition, the disruption of the *HAC1* gene slightly reduced the specific glucose consumption rate from 0.90 to 0.72 g g^{-1} h^{-1}, resulting in retarded growth in the *hac1Δ* mutant. However, the *ire1Δ* strain showed the growth pattern similar to the wild type control strain on YEPDG medium (Fig. 2C) although it expressed a decreased level of LK8 (Fig. 1). This indicates that galactose utilization in the LK8-producing cells may not be associated with the activation of Ire1p since the *ire1Δ* strain has exhibited normal growth and carbon utilization patterns.

Two forms of Hac1p: the induced (spliced, Hac1p^i) and uninduced (unspliced, Hac1p^u) versions were used for complementation experiments. The *HAC1*^{i} and the *HAC1*^{u} genes were expressed by the *GAL1* promoter in the *HAC1*-disrupted host strain.

The growth patterns of four strains, YTL30 (WT), YTL59 (*hac1Δ*), YTL63 (*hac1Δ HAC1*^{i}) and YTL64 (*hac1Δ HAC1*^{u}) were tested on YEPG plates (Fig. 3A). The strain YTL59 did not grow on the YEPG plate, as expected from the previous batch fermentation (Fig. 2B). In contrast to the negative control strain (YTL59), YTL63 and YTL64 strains were able to grow on YEPG media. However, their growth phenotypes were partly restored and the two strains showed smaller colonies than the positive control strain (YTL30). To assess the accurate growth patterns accurately, liquid cultivations in YEPG media were performed with these four strains (Fig. 3B).

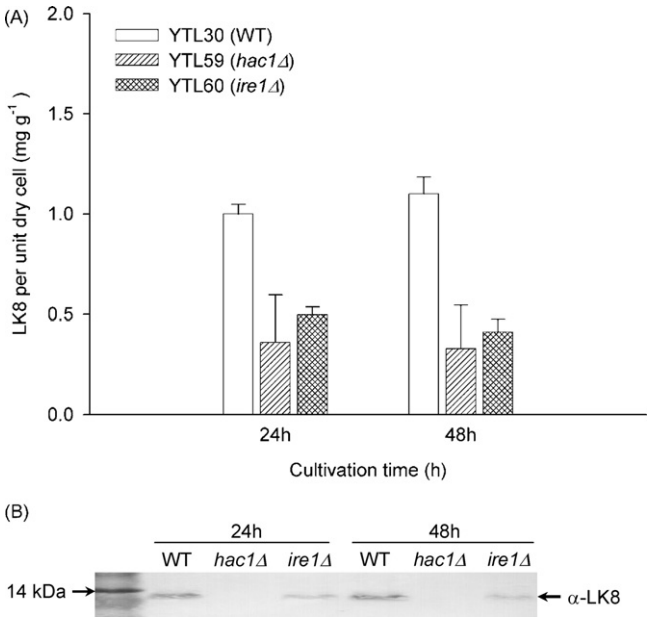

Fig. 1. Specific LK8 contents produced by three strains of YTL30 (WT, open), YTL59 (*hac1Δ*, hatched), and YTL60 (*ire1Δ*, coarse) in batch fermentations at 24 h and 48 h. The levels of secreted LK8 were determined in direct ELISA (A). The intracellular levels of LK8 trapped in cells were relatively analyzed in an immunoblot done with anti-LK8 primary antibody (1:2000) (B).

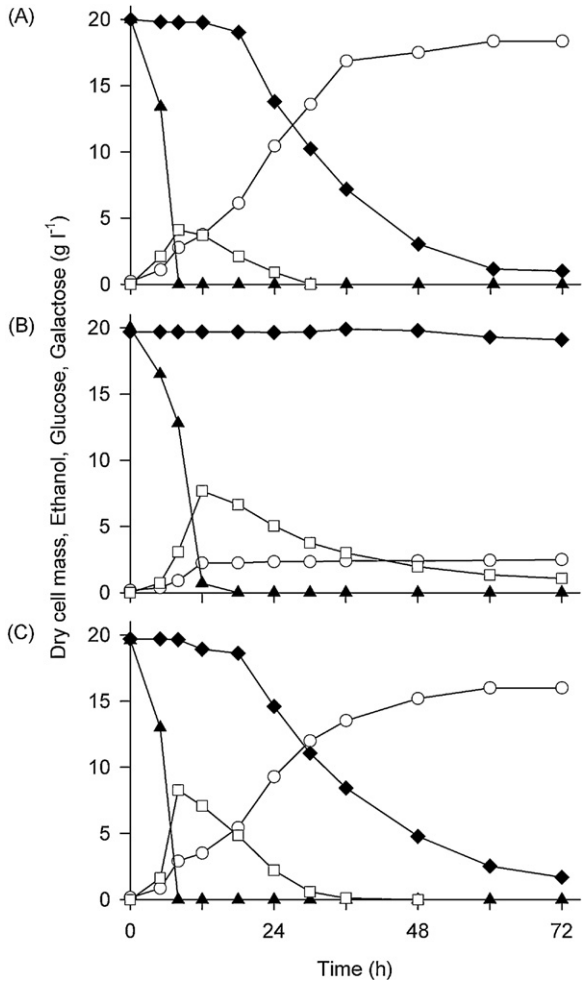

Fig. 2. Changes in concentration of cell growth (○), ethanol (□), glucose (▲) and galactose (♦) in batch fermentations of YTL30 (WT, A), YTL59 (*hac1Δ*, B), and YTL60 (*ire1Δ*, C).

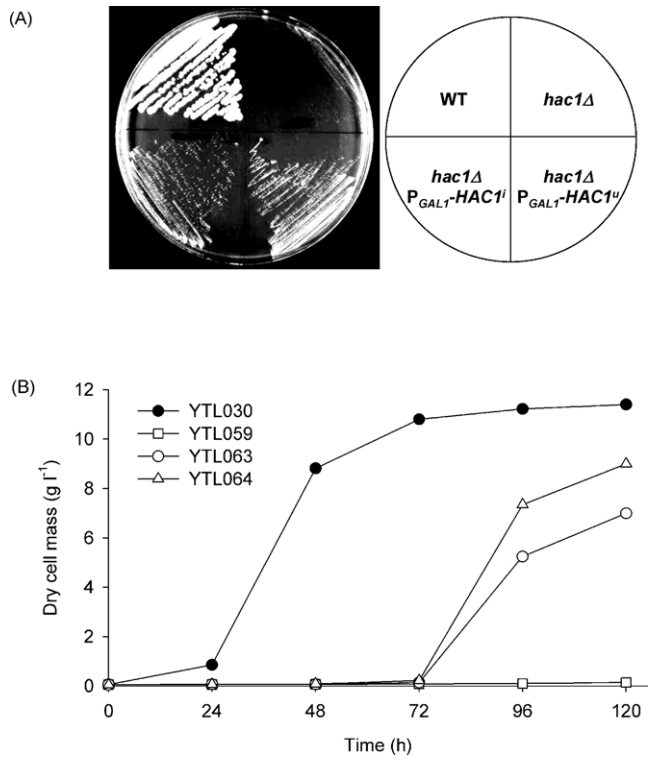

Fig. 3. Recovery of growth for recombinant *S. cerevisiae* strains which expressed the Hac1p^{i} and the Hac1p^{u} under the control of *GAL1* promoter. YTL63 (*hac1Δ* P_{GAL1}-*HAC1*^{i}) and YTL64 (*hac1Δ* P_{GAL1}-*HAC1*^{u}) compared with YTL30 (WT, positive control) and YTL59 (*hac1Δ*, negative control). Freshly grown strains in SC uracil dropout media supplemented 0.3 mM of myo-inositol were used. Plate assay (A) was performed with YEPG plate (yeast extract 1%, peptone 2% and galactose 2%). Cells were grown (B) were obtained from 100 ml flask cultivation at 30 °C in liquid YEPG media and the optical density (OD_{600}) for yeast cells at initial time was adjusted to 0.5 after the inoculums.

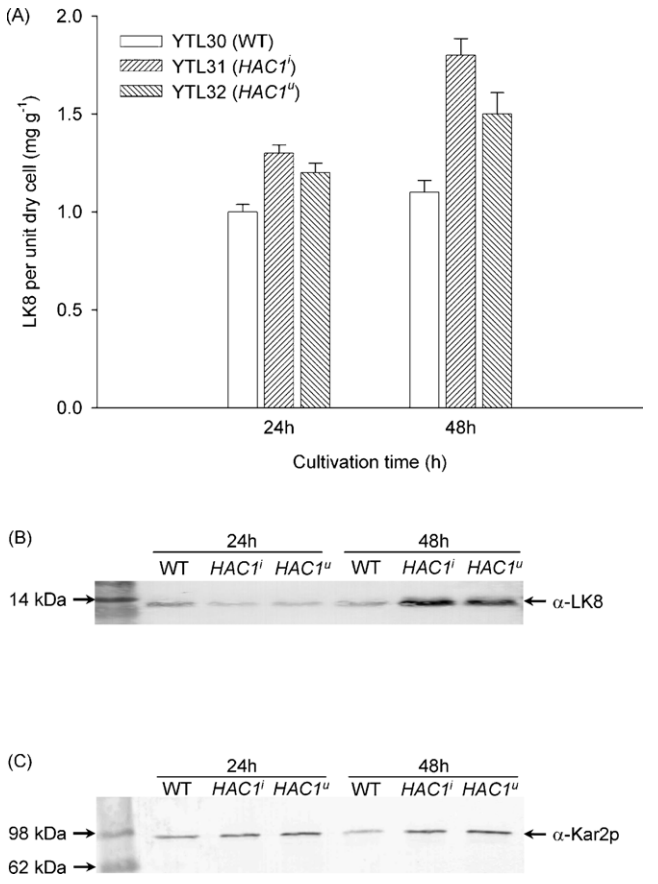

Fig. 4. Secreted (extracellular) and intracellular specific LK8 contents produced by three strains of YTL30 (WT, open), YTL31 (*HAC1*^{i}, hatched) and YTL32 (*HAC1*^{u}, back-hatched). Secretion levels were quantitatively analyzed by direct ELISA (A). Intracellular portions of LK8 were detected by immunoblot with the anti-LK8 primary antibody (1:2000) (B). Kar2p changes in YTL30 (WT), YTL31 (*HAC1*^{i}), YTL32 (*HAC1*^{u}) strains with anti-Kar2p antibodies (C).

As expected, YTL63 and YTL64 showed a growth lag longer for two days than the YTL30 control strain. However, the YTL63 and YTL64 mutants grew as high as 61% and 79% of the maximum dry cell mass of the control strain, respectively. The results indicate that the Hac1p is required for cell growth and galactose consumption of the LK8-producing *hac1Δ* mutant.

3.3. Effects of overexpression of HAC1 gene on LK8 expression level

To examine the effect of overexpression of the *HAC1* gene on LK8 protein production, YTL31 and YTL32 strains were constructed and LK8 production levels were compared in batch fermentations. The values of maximum dry cell mass for YTL30, YTL31 and YTL32 strains reached 18, 15 and 16 g l^{-1}, respectively (data not shown). Specific growth rates measured in the exponential growth phase were around 0.054–0.056 h^{-1} in YEPDG, indicating that no clear difference in cell growth among these strains in the batch fermentations. Likewise, specific rates of glucose and galactose consumption for the three recombinant yeast strains exhibited almost the same values (data not shown). However, the levels of secreted LK8 at 48 h increased by a 1.6-fold for YTL31 and a 1.4-fold for YTL32 compared with that of YTL30 (Fig. 4A).

For the samples taken at 48 h for YTL31 and YTL32, the intracellular LK8 level increased by a 4-fold (Fig. 4B). To confirm the overexpression of the *HAC1* gene, immunoblot was performed using the anti-Kar2p antibody (Fig. 4C). The expression levels of Kar2p detected in the immunoblot for YTL31 and YTL32 were elevated by 2.3- and 2.7-folds, respectively, compared with the wild type control strain. These results clearly suggested that overexpression of the *HAC1* gene was effective in inducing UPR. The *GAL1* promoter driven induction of Hac1p^{i} and Hac1p^{u} significantly enhanced both the intracellular and secreted production of LK8 in batch fermentations.

4. Discussion and conclusions

This work has demonstrated that heterologous protein production was greatly dependent on Ire1p and Hac1p. Disruption of the *IRE1* gene inactivated the induced pathway of UPR, resulting in reduced secretion and intracellular expression of LK8. The same result was also observed by disruption of the *HAC1* gene (Fig. 1). It has been known that the inactivation of Ire1p ribonuclease failed to perform the cleavage step in the splicing of mRNA encoding Hac1p, the transcription factor that activates gene expression in UPR [19,28,29]. The experiment results in this study suggested that Hac1p activated by the specific splicing reaction was essential, thereby allowing the cells to efficiently synthesize the heterologous protein, dependent on the *HAC1* and *IRE1* genes. Intriguingly, the assimilation of galactose as a carbon source for cell growth as well as an inducer for LK8 production was regulated in an *IRE1*-independent and *HAC1*-dependent manner as shown in Fig. 2. This indicated that the unspliced Hac1p (Hac1p^{u}) activated a specific mechanism for carbon uptake, independent of Ire1p. An expression problem was also observed in ethanol and galactose uptake in the *hac1Δ* strain (Fig. 2B). It is supposed to show a “diauxic growth”

pattern (Fig. 2A and C) by utilizing ethanol or galactose as the substrates. However, the diauxic growth was not seen in the *hac1Δ* strain (Fig. 2B). Therefore, the strain showed no more growth and retarded consumption of ethanol and galactose in 12 h of fermentation time. It might be due to the fact that the genes necessary for maintenance of homeostasis were not able to be expressed by the deactivation of UPR. This might imply that *IRE1*-independent uninduced Hac1p has a role for maintaining viability in the cells able to overexpress heterologous proteins. However, the *HAC1*^{u} mRNA which potently encodes a 230-amino acid protein was translationally inactive due to the presence of the inhibitory intron, thereby no traceable amount of Hac1p^{u} was detected *in vivo* [17]. To produce the active form of Hac1p, the *HAC1* mRNA must be spliced in response to ER stress [30]. Mori and coworkers have argued the role of the unspliced transcript of the *HAC1* gene encoding 230-amino acid Hac1p which could suppress the inositol requirement by loss of Hac1p despite of its low transcriptional activity of UPR [31]. Furthermore, they proposed that the *HAC1* promoter itself responded to ER stress to induce transcription of its downstream genes, similar to an UPRE (UPR element)-like sequence of the *KAR2* promoter which represented a major target of the UPR [32]. To this end, both unspliced and spliced forms of Hac1p were expressed under the control of the *GAL1* promoter in the *HAC1*-disrupted strain, resulting in partial growth recovery in a medium containing galactose as the complementation experiment (Fig. 3). It was shown that the *HAC1* gene was still necessary for normal cell growth even in the *ire1Δ* strain as if the Hac1p^{u} was moderately produced *in vivo*, with no aid of Ire1p. As previously discussed [32], cells have a mechanism for increasing the amount of Hac1p^{u} translated, independent of the activation of Ire1p. There was another report that Rlg1p has a dual function of translation regulation of HAC1 5′ UTR as well as tRNA ligase activity [33]. To date, the mechanism of Hac1p^{u} translation has not been clearly understood. However, it is obvious that the *HAC1* gene is required for maintaining the homeostasis in heterologous protein overexpression in yeasts.

Spear and coworkers [34], on the other hand, demonstrated the *ire1Δ* strain was lethal in a medium with 2% galactose when producing carboxypeptidase Y (CPY), although the *ire1Δ* mutant in this work readily grew in the medium. This contradiction could be explained by a difference in intrinsic toxicity of accumulated model proteins. LK8 has smaller molecular weight (9.6 kDa) than mature CPY (61 kDa) and it was easy to be secreted from the cells. Moreover, accumulation of unfolded proteins alone was sometimes insufficient to trigger the classical UPR pathway since the splicing of *HAC1* mRNA could be led by the blockages in ER-distal compartments of the secretion pathway [35]. Activation of UPR may be initiated differently in which the cells produced various kinds of foreign proteins or faces different circumstances to cope with. Meanwhile, in the experiments tested with the *hac1Δ*, *ire1Δ* mutants and appropriate control strains containing a 2 μ-based LK8 expression vector [24], all strains grew well and no growth defect was observed for the relevant strains episomally expressing LK8 in YEPG media (data not shown). Consequently, the degree of intrinsic toxicity was dependent the dosage of a target gene. The *hac1Δ* and *ire1Δ* strains were only viable if the strains had relatively low copies of the LK8 gene or an additional copy of Hac1p in a host *S. cerevisiae* strain.

In both intracellular and secreted parts, a specific content of LK8 increased by the *GAL1* promoter controlled coexpression of the *HAC1*^{i} and *HAC1*^{u} genes (Fig. 4). It was suggested that overexpression of Hac1p elevated not only a secreted level but also the total expression level of LK8. From our previous studies, overexpression of Kar2p or Pdi1p increased secretion of a model heterologous protein [10,11] by reducing the fraction of the trapped LK8 inside the cells (unpublished data). Taken together, a bottleneck of the secretory machinery for heterologous proteins could be the folding pathway including the mechanism with Kar2p and Pdi1p rather than the UPR pathway modulated by the action of Hac1p [36]. As the additional activation of UPR reinforces the total expression level of LK8, the simultaneously expression of Hac1p and Pdi1p seems to improve secretion of the heterologous protein.

In conclusion, functions of Hac1p and Ire1p are sufficient and necessary for production of heterologous proteins and normal cell growth under the UPR condition. It was observed that the single-copy overexpression of the *HAC1* gene elevated the total expression of LK8. Thus, it can be concluded that the Hac1p overexpression is needed for improving the expression level of a target protein in recombinant *S. cerevisiae*.

Acknowledgements

This research was supported by Advanced Biomass R&D Center (ABC) of Korea Grant (2010-0029799) funded by the Ministry of Education, Science and Technology and Korea Research Council of Fundamental Science & Technology (KRCF) grant.

References

[1] Sarramegna V, Talmont R, Demange P, Milon A. Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cell Mol Life Sci 2003;60:1529–46.
[2] Báez J, Olsen D, Polarek JW. Recombinant microbial systems for the production of human collagen and gelatin. Appl Microbiol Biotechnol 2005;69:245–52.
[3] Jahic M, Veide A, Charoenrat T, Teeri T, Enfors SO. Process technology for production and recovery of heterologous proteins with *Pichia pastoris*. Biotechnol Progr 2006;22:1465–73.
[4] van Ooyen AJJ, Dekker P, Huang M, Olsthoorn MMA, Jacobs DI, Colussi PA, et al. Heterologous protein production in the yeast *Kluyveromyces lactis*. FEMS Yeast Res 2006;6:381–92.
[5] Morlino GB, Tizzani L, Fleer R, Frontali L, Bianchi MM. Inducible amplification of gene copy number and heterologous protein production in the yeast *Kluyveromyces lactis*. Appl Environ Microbiol 1999;65:4808–13.
[6] Hollenberg CP, Gellissen G. Production of recombinant proteins by methylotrophic yeasts. Curr Opin Biotechnol 1997;8:554–60.
[7] Schuller D, Casal M. The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry. Appl Microbiol Biotechnol 2005;68:292–304.
[8] Kim E-J, Park Y-K, Lim H-K, Park Y-C, Seo J-H. Expression of hepatitis B surface antigen S domain in recombinant *Saccharomyces cerevisiae* using *GAL1* promoter. J Biotechnol 2009;141:155–9.
[9] Lee FWF, DaSilva NA. Improved efficiency and stability of multiple cloned gene insertions at the delta sequences of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 1997;48:339–45.
[10] Cha KH, Kim MD, Lee TH, Lim HK, Jung KH, Seo JH. Coexpression of protein disulfide isomerase (PDI) enhances production of kringle fragment of human apoliprotein(a) in recombinant *Saccharomyces cerevisiae*. J Microbiol Biotechnol 2006;16:308–11.
[11] Kim MD, Han KC, Kang HA, Rhee SK, Seo JH. Coexpression of BiP increased antithrombotic hirudin production in recombinant *Saccharomyces cerevisiae*. J Biotechnol 2003;101:81–7.
[12] Mattanovich D, Gasser B, Hohenblum H, Sauer M. Stress in recombinant protein producing yeasts. J Biotechnol 2004;113:121–35.
[13] Bernales S, Papa FR, Walter P. Intracellular signaling by the unfolded protein response. Annu Rev Cell Dev Biol 2006;22:487–508.
[14] Ma YJ, Hendershot LM. The unfolding tale of the unfolded protein response. Cell 2001;107:827–30.
[15] Fewell SW, Travers KJ, Weissman JS, Brodsky JL. The action of molecular chaperones in the early secretory pathway. Annu Rev Genet 2001;35:149–91.
[16] Park Y-K, Jung S-M, Lim H-K, Son Y-J, Park Y-C, Seo J-H. Effects of Trx2p and Sec23p expression on stable production of hepatitis B surface antigen S domain in recombinant *Saccharomyces cerevisiae*. J Biotechnol 2012;160:151–60.
[17] Cox JS, Walter P. A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. Cell 1996;87:391–404.
[18] Okamura K, Kimata Y, Higashio H, Tsuru A, Kohno K. Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. Biochem Biophys Res Commun 2000;279:445–50.
[19] Sidrauski C, Walter P. The transmembrane kinase Ire1p is a site-specific endonuclease that initiates mRNA splicing in the unfolded protein response. Cell 1997;90:1031–9.
[20] Guo J, Polymenis M. Dcr2 targets Ire1 and downregulates the unfolded protein response in *Saccharomyces cerevisiae*. EMBO Rep 2006;7:1124–7.
[21] Kaufman RJ, Scheuner D, Schroder M, Shen XH, Lee K, Liu CY, et al. The unfolded protein response in nutrient sensing and differentiation. Nat Rev Mol Cell Biol 2002;3:411–21.
[22] Valkonen M, Penttilä M, Saloheimo M. Effects of inactivation and constitutive expression of the unfolded-protein response pathway on protein production in the yeast *Saccharomyces cerevisiae*. Appl Environ Microbiol 2003;69:2065–72.

[23] Breinig F, Diehl B, Rau S, Zimmer C, Schwab H, Schmitt MJ. Cell surface expression of bacterial esterase A by *Saccharomyces cerevisiae* and its enhancement by constitutive activation of the cellular unfolded protein response. Appl Environ Microbiol 2006;72:7140–7.
[24] Cha KH, Kim MD, Lee TH, Lim HK, Jung KH, Seo JH. Selection of optimum expression system for production of kringle fragment of human apolipoprotein(a) in *Saccharomyces cerevisiae*. Biotechnol Bioprocess Eng 2004;9:523–7.
[25] Kim JS, Yu HK, Ahn JH, Lee HJ, Hong SW, Jung KH, et al. Human apolipoprotein(a) kringle V inhibits angiogenesis in vitro and in vivo by interfering with the activation of focal adhesion kinases. Biochem Biophys Res Commun 2004;313:534–40.
[26] Lee TH, Kim MD, Seo JH. Development of reusable split *URA3*-marked knockout vectors for *Saccharomyces cerevisiae*. J Microbiol Biotechnol 2006;16:979–82.
[27] Burke D, Dawson D, Stearns T. Methods in yeast genetics. New York, NY: Cold Spring Harbor Laboratory Press; 2000.
[28] Gonzalez TN, Sidrauski C, Dorfler S, Walter P. Mechanism of non-spliceosomal mRNA splicing in the unfolded protein response pathway. EMBO J 1999;18:3119–32.
[29] Kaufman RJ. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 1999;13:1211–33.
[30] Chapman RE, Walter P. Translational attenuation mediated by an mRNA intron. Curr Biol 1997;7:850–9.
[31] Mori K, Ogawa N, Kawahara T, Yanagi H, Yura T. mRNA splicing-mediated C-terminal replacement of transcription factor Hac1p is required for efficient activation of the unfolded protein response. Proc Natl Acad Sci U S A 2000;97:4660–5.
[32] Ogawa N, Mori K. Autoregulation of the *HAC1* gene is required for sustained activation of the yeast unfolded protein response. Genes Cells 2004;9:95–104.
[33] Mori T, Ogasawara C, Inada T, Englert M, Beier H, Takezawa M, et al. Dual functions of yeast tRNA ligase in the unfolded protein response: unconventional cytoplasmic splicing of HAC1 pre-mRNA is not sufficient to release translational attenuation. Mol Biol Cell 2010;21:3722–34.
[34] Spear ED, Ng DTW. Stress tolerance of misfolded carboxypeptidase Y requires maintenance of protein trafficking and degradative pathways. Mol Biol Cell 2003;14:2756–67.
[35] Leber JH, Bernales S, Walter P. IRE1-independent gain control of the unfolded protein response. PLoS Biol 2004;2:1197–207.
[36] Kim JH, Zhao Y, Pan X, He X, Gilbert HF. The unfolded protein response is necessary but not sufficient to compensate for defect in disulfide isomerization. J Biol Chem 2009;284:10400–8.