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# Expression and Secretion of Human Serum Albumin in the Yeast *Saccharomyces cerevisae*

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**Abstract** In order to maximize the secretory expression of human serum albumin (HSA) in the yeast *Saccharomyces cerevisiae*, a series of HSA expression vectors were constructed with a combination of different promoters, 5' untranslated regions (5'UTR), and secretion signal sequences. The expression vector composed of the galactose-inducible promoter *GAL10*, the natural 5'UTR, and the natural signal sequence of HSA directed the most efficient expression and secretion of HSA among the constructed vectors when introduced into several *S. cerevisiae* strains. Although the major form of HSA expressed and secreted in the yeast transformants was the mature form of 66 kDa, the truncated form of 45 kDa was also detected both in the cell extract and in the culture supernatant. The level of the intact HSA protein in the culture supernatant reached up to 30 mg/l at 24 h of cultivation in a shake-flask culture but began to decrease afterwards, indicating that the secreted HSA protein was unstable in a prolonged culture of yeast.

**Key words:** *Saccharomyces cerevisiae*, human serum albumin, secretion

Human serum albumin (HSA) is a single chain non-glycosylated polypeptide composed of 585 amino acids with a molecular weight of 66.6 kDa. It is the most abundant plasma protein, constituting approximately 60% of the total protein content in plasma [17]. The protein may be found in most body fluids, where it functions as a soluble carrier for the transport of fatty acids, bile pigments, amino acids, steroid hormones, and metal ions. HSA is clinically in great demand to be used as a replacement fluid of plasma expander or as a compensator for blood losses. Due to its pharmaceutical importance, the expression of HSA in a variety of microorganisms has been attempted to produce large quantities of the recombinant HSA free from contamination by human pathogens [9]. Compared to the inclusion body formation and the poor secretion of recombinant HSA in *Escherichia coli* [13] and *Bacillus subtilis* [19], respectively, the efficient secretion of a correctly folded and processed recombinant HSA has been reported in several yeast systems including *Sacchromyces cerevisiae* [21, 16], *Kluyveromyces lactis* [6], and *Pichia pastoris* [22].

When a recombinant protein is expressed in a heterologous host, problems can arise at numerous stages from transcription through to protein stability. Although the ultimate level of expression of each protein is largely dependent on its inherent properties which present its own unique set of problems [8], the expression level can be improved by adjusting one or more parameters in the expression vector, culture medium, and growth conditions. Therefore, the design of an expression vector optimized in the configuration of regulatory sequence, protein coding sequence, and its flanking sequence is an important initial step to achieving the high-level production of a recombinant protein.

We present here the secretory expression of recombinant HSA in the yeast *S. cerevisiae*, which has been regarded as a particularly attractive host system for the production of human therapeutic proteins due to its GRAS (generally recognized as safe) status [1]. We constructed a series of expression vectors to optimize the expression and secretion of HSA in *S. cerevisiae* and investigated several factors affecting the expression level of the recombinant HSA, such as promoter strength, mRNA stability, translation efficiency, protein secretion efficiency, and protein stability.

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## MATERIALS AND METHODS

### Strains and Plasmids

Yeast strains used as the host for the expression of HSA were *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb-Δ1.6R can1 his3-20 ura3-52*), L3262 (*MATa ura3-52 leu2-3,112 his4-34*), and 334 (*MATα pep4-3 prb1-1122 ura3-52 leu 2-3,112 reg1-501 gal1*).

The yeast vector YEGα-HIR525 has been described previously [2]. The vector pGAPD-HIR (Sohn J.-H., unpublished result) is identical to YEGα-HIR525 except that it has the *GAPD* (glyceraldehyde-3-phosphate dehydrogenase) promoter instead of the *GAL10* promoter. The yeast expression vectors pYH-10Zd and its derivative pYH-FZd contain the *PGK* (phosphoglycerate kinase) promoter and the *CUP1* promoter, respectively [3].

### The cDNA of HSA

The plasmid pBlue-HSAI, which has the 2.2-kb HSA cDNA fragment containing the 5' untranslated region (5'UTR), the whole coding region of HSA, and the 3'UTR of HSA in the *Eco*RI site of pBluescriptSK (Stratagene, La Jolla, CA, U.S.A.), was obtained from the cDNA library of human fetal liver (provided by Dr. Choi, I. S. at the Korea Research Institute of Bioscience and Biotechnology). The entire cDNA was sequenced to confirm that the amino acid sequence deduced from the cDNA fragment was identical to that of normal albumin A [14]. To remove the natural 5'UTR of HSA, the 52-bp *Eco*RI/*Bst*EII fragment of the HSA cDNA containing the natural 5'UTR of HSA was exchanged with the 24-bp *Eco*RI/*Bst*EII oligonucleotide containing the *Eco*RI site at three bases upstream of the start codon of HSA cDNA (Fig. 1). The 24-bp *Eco*RI/*Bst*EII oligonucleotide was ligated with the ~2.0-kb *Bst*EII/*Hin*dIII HSA cDNA fragment from pBlue-HSAI and then cloned between the *Eco*RI and *Hin*dIII sites of pBluescriptKS, generating pBlue-HSAII that contained the HSA cDNA without the natural 5'UTR. The natural secretion signal sequence of HSA was exchanged with the signal sequence of 23 amino acids derived from inulinase of *Kluyveromyces marxianus* (Fig. 2). The 72-bp *Eco*RI/*Ase*I DNA fragment containing 20 amino acids of the inulinase signal sequence was ligated with the 0.2-kb *Ase*I/*Afl*III HSA cDNA fragment which encodes the mature HSA and the last three amino acids of the inulinase signal sequence fused in frame to the N-terminal fragment of mature HSA, resulting a 0.3-kb *Eco*RI/*Afl*III fragment. The *Ase*I/*Afl*III cDNA fragment was obtained by polymerase chain reaction using the HSA (*Ase*I) primer (5'GGGATTAATTACAAGAGAGATGCACACAAGAGTG3') and the HSA (*Afl*III) primer (5'GCTGACTCATCAGCAACAC3'). The 0.3-kb *Eco*RI/*Afl*III fragment was then ligated with the 1.7-kb *Afl*III/*Hin*dIII of HSA cDNA and inserted between the *Eco*RI and *Hin*dIII sites of pBluescriptKS, generating pBlue-InuHSA which contained the cDNA of mature HSA fused with the inulinase signal sequence.

### Construction of HSA Expression Vectors

To construct HSA expression vectors for *S. cerevisiae* (Fig. 3), the 2.0-kb *Eco*RI/blunt ended *Hin*dIII fragment containing the 5'UTR and the whole coding region of HSA was obtained from pBlue-HSAI and ligated with the 6.5-kb *Eco*RI/blunt ended *Sal*I fragment derived from pGAPD-HIR or the 6.0-kb *Eco*RI/blunt ended *Sal*I fragment from YEGα-HIR525, resulting in the expression vectors pYHSA1 and pYHSA5, respectively. The 2.0-kb *Eco*RI/*Sal*I HSA cDNA fragment without the 5'UTR, generated from pBlue-HSAII, was ligated with the 6.5-kb *Eco*RI/*Sal*I fragment from pGAPD-HIR, resulting in the expression vector pYHSA2. The 2.0-kb *Eco*RI/*Sal*I fragment encoding the Inu-HSA fusion protein was obtained from pBlue-InuHSA and ligated with the 6.5-

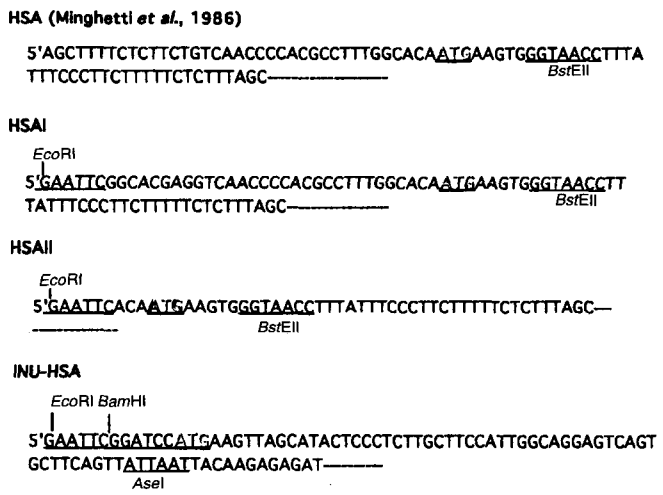


**Fig. 1.** The 5' untranslated region of various human serum albumin cDNAs under this study.

The nucleic acid sequences of 5'UTR in the HSA cDNA reported previously [14], the HSA cDNA cloned in this study (HSAI), the cDNA devoid of the natural 5'UTR (HSAII), and the cDNA encoding the mature HSA fused with the inulinase signal sequence (Inu-HSA) are shown. The translation initiation codon ATG is highlighted with double-line letters.

**Fig. 2.** Primary structure of the secretory leader sequences described in this study.

A, Prepro leader sequence of HSA (24 a.a.); B, Prepro leader sequence of *K. marxianus* inulinase (23 a.a.). The putative cleavage sites for signal peptide (△) and a cleavage site for KEX2 (↑) are indicated.

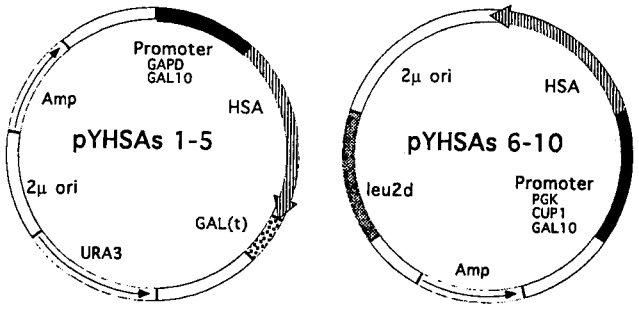


**Fig. 3.** Generic plasmid base for the expression of HSA from *S. cerevisiae.*

The expression vectors are *E. coli*-yeast shuttle vectors based on 2 μ plasmid. The plasmids pYHSA1 to pYHSA5 contain the *URA3* gene and the *GAL7* terminator. The plasmids pYHSA6 to pYHSA10 contain the *leu2-d* gene and the *FLD* gene terminator that is provided by the 2 μ sequence [23].

kb *Eco*RI/*Sal*I fragment of pGAPD-HIR, leading to the expression vectors pYHSA3. The vectors pYHSA6 and pYHSA7 were constructed by ligating the 2.0-kb *Eco*RI/ blunt ended *Hin*dIII fragments of HSA cDNA, obtained from pBlueHSAI or from pBlueHSAII, with the 8-kb *Sac*I/blunt ended *Bam*HI fragment of pYH-10Zd. pYHSA8 was made by ligating the 2.0-kb *Eco*RI/blunt ended *Hin*dIII fragment of HSA cDNA from pBlueHSAI with the 8-kb *Sac*I/blunt ended *Bam*HI fragment of pYH-FZd. The plasmid pYHSA10 was generated by blunt-end ligation of the 3-kb *Bam*HI/*Hin*dIII fragment from pYHSA5, which contains the *GAL10* promoter and the HSA cDNA, with the 7-kb *Sal*I/*Sac*I fragment from pYH-FZd.

### Media and Culture Conditions

In shake-flask culture, triple-baffled 250-ml capacity shake flasks were used to ensure adequate mixing and oxygen transfer. Duplicate flasks containing 25-ml growth medium were inoculated with pre-cultured cells in SD-URA, a synthetic complete medium without uracil [20], and incubated with vigorous shaking at 30°C. For expression of HSA from the *GAPD* and *PGK* promoters, YPD (1% yeast extract, 2% peptone, 2% dextrose) was used as a growth medium. To activate the *CUP1* promoter, 0.03 mM copper sulfate was added to the YPD medium. For induction from the *GAL10* promoter, YPDG (1% yeast extract, 2% peptone, 1% dextrose, 2% galactose) was used. In the case of galactose feeding, yeast cells were cultured in YPDG for 24 h and then fed with galactose every 12 h up to 72 h to restore the concentration of galactose at 2% in the medium.

### Western Blot Analysis

Western blot analysis of the total yeast cell lysates, obtained as described previously (12), and the culture supernatant of yeast cells was carried out with the polyclonal antibody raised against human serum albumin (Sigma St. Louis, MO, U.S.A.). The color development of the peroxidase reaction was carried out using 3,3'-5,5'-tetramethylbenzidene stabilized substrate (Promega, Madison, WI, U.S.A.). The amount of HSA in the culture supernatant was estimated by densitometric tracing of 66-kDa HSA band in Western blot with the authentic HSA (Sigma) as a standard.

### Northern Blot Analysis

Yeast total RNA was prepared by the hot phenol extraction method of Elion and Warner [5], fractionated on a 1.2% formaldehyde-agarose gel and then blotted onto Qiabrane membrane (Qiage, Valencia, CA, U.S.A.). The blot was probed with the 1.8-kb long digoxigenin-labeled HSA cDNA fragment. Colorimetric detection of the membrane was performed with the reaction of anti-digoxigenin alkaline phosphatase (Boehringer Mannheim Mannheim, Germany)

## RESULTS AND DISCUSSION

### Effect of Promoter and Selection Marker on the Expression of HSA

In constructing a series of expression vectors of human serum albumin (pYHSA series) for the yeast *S. cerevisiae*, we systematically examined the effect of various vector components, such as promoter, 5'UTR, signal sequence, and selection marker, on the expression of HSA in *S. cerevisiae*. Since gene expression is most frequently regulated at the level of transcription, we first evaluated various yeast promoters in directing the expression of HSA. The promoters tested in this study were two constitutive expression promoters (the *GAPD* and *PGK* promoters) and two inducible promoters (the galactose-regulated *GAL10* and cupper-regulated *CUP1* promoters), which are known to drive efficient transcription of a variety of heterologous genes in yeast [18]. Among the promoters employed in constructing the HSA expression vectors, the galactose-inducible *GAL10* promoter appeared to produce higher expression of HSA than any other promoters in two different vector backgrounds (Table 1). At least two or threefold higher levels of HSA expression were observed with the *GAL10* promoter as compared to the other promoters.

The HSA expression vectors constructed in this work are based on yeast 2 μ replication origin, which ensures the relatively stable maintenance of expression vectors in high copy number [18]. As an approach to further increasing the stability and copy number of the expression vectors, the auxotrophic selectable marker *leu2-d*, a poorly expressed allele of *LEU2* [7], was used instead of the marker *URA3* in constructing another set

**Table 1.** Effect of promoter and selection marker on the expression of HSA in *S. cerevisiae*.

| Plasmid | Promoter | 5'UTR/ Signal[a] | Marker | Yield (mg/l)[b] |
|---|---|---|---|---|
| pYHSA1 | GAPD | H | *URA3* | 10 |
| pYHSA5 | GAL10 | H | *URA3* | 20 |
| pYHSA6 | PGK | H | *leu2-d* | 10 |
| pYHSA8 | CUP1 | H | *leu2-d* | 10 |
| pYHSA10 | GAL10 | H | *leu2-d* | 20 |

[a]The 5'UTR and secretion signal sequence was derived from the cDNA of HSA1 (Fig. 1). [b]The level of HSA in the culture supernatant of *S. cerevisiae* L3262 transformants was determined after 1-day cultivation either in YPD or YPDG medium by densitometric tracing of 66 kDa of HSA band in Western blot. Values represent the means for duplicated cultures of two individual transformants.

**Table 2.** Effect of 5'UTR and signal sequence on the HSA expression in *S. cerevisiae*.

| Plasmid | Promoter | HSA-5'UTR | Signal[a] | Yield (mg/l)[b] |
|---|---|---|---|---|
| pYHSA1 | GAPD | + | H | 10 |
| pYHSA2 | GAPD | – | H | 5 |
| pYHSA3 | GAPD | – | INU | 3 |
| pYHSA6 | PGK | + | H | 10 |
| pYHSA7 | PGK | – | H | 8 |

[a]Secretion signal sequences: Sequence from humun serum albumin (H) and from *Kluyveromyces* inulinase (INU). [b]The level of HSA in the culture supernatant of *S. cerevisiae* L3262 transformants after 1-day cultivation was determined by densitometric tracing of 66 kDa of HSA band in Western blot.

of HSA expression vectors (Fig. 3). The vectors containing the defective *leu2-d* allele would be expected to be present in very high copy number for *leu2* strains to grow in leucine-deficient medium [23]. However, compared to the *URA3* vector pYHSA5, the *leu2-d* vector pYHSA10 did not improve the expression level of HSA (Table 1). Southern blot analysis of the total DNA of the yeast transformants revealed that the copy number of the *leu2-d* vector was just about 20~30 copies per cell even in leucine-deficient medium, which was comparable to that of the *URA3* vector (data not shown). It is not clear at present why the copy number of the *leu2-d* vector pHYSA10 did not increase to the high levels previously expected.

### Effect of 5'UTR and Signal Sequence on the Expression of HSA

The nucleotide composition and length of the mRNA 5'UTR region may influence expression levels of proteins by affecting either mRNA stability or translation efficiency. It is generally assumed that a G, C, or G+C rich leader of higher eukaryotic cDNA could have an inhibitory effect on the translational expression of foreign genes in yeast [4]. To investigate the possible detrimental effect exerted by the 5'UTR derived from the HSA cDNA (Fig. 1), some of the expression vectors devoid of the HSA 5'UTR were constructed (Table 2). The presence of the natural 5'UTR of HSA in the expression vectors, however, did not show any appreciable inhibition effect. Rather, the presence of the 5'UTR showed an enhancing effect on the expression of HSA from the *GAPD* or the *PGK* promoter (Table 2). The involvement of 5'UTR in albumin gene expression had been previously implied based on the observation that the 5'UTR of albumin mRNA was quite conserved among human, rat, and chicken albumin genes and that the 5'UTR can make hypothetical interaction with the highly conserved 18S rRNA (14). Northern blot analysis of HSA mRNA from the recombinant yeast strains

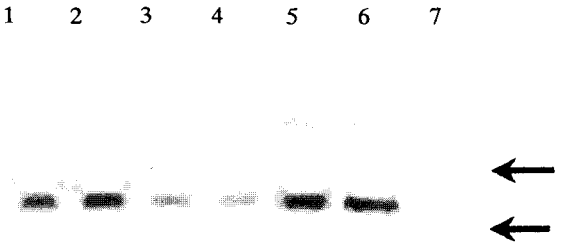


**Fig. 4.** Northern blot analysis of HSA transcripts expressed in *S. cerevisiae* transformants.
Total RNA were prepared from the two individual transformants harboring pYHSA3 (lanes 1 and 2), pYHSA2 (lanes 3 and 4), pYHSA1 (lanes 5 and 6), and pGAPD-HIR (lane 7), respectively, which were cultured overnight in YPD medium. 20 μg of RNA samples were fractionated on 1.2% formaldehyde agarose gel, blotted to membrane, and hybridized with the 1.8-kb digoxigenin-labeled *Bst*EII/*Hin*dIII HSA cDNA fragment. Arrows indicated the positions of 18S (1.8 kb) and 25S (3.2 kb) yeast ribosomal RNA.

harboring pYHSA1 or pYHSA2 (Fig. 4) revealed that the expression levels of HSA protein were well correlated with those of HSA mRNA with a length of ~2.5 kb. This result indicates that the presence of the 5'UTR somehow increased HSA mRNA stability, thus exerting a positive effect on the expression of HSA.

Since heterologous protein secretion is often more efficient when secretion signals from yeast are used, the inulinase signal sequence of *K. marxianus* was tested in directing the secretion of HSA from *S. cerevisiae*. The signal sequence has recently proven efficient for the secretion of heterologous proteins with a large molecular weight in yeast [12]. In directing the secretion of HSA in *S. cerevisiae*, the inulinase signal sequence appeared to be as efficient as the natural HSA sequence since less

than 10% of total HSA expressed was retained intracellularly as in the case of HSA secretion with the natural signal sequence (data not shown). However, the final amount of HSA present in the culture supernatant was much lower when the inulinase sequence was used compared to when the natural HSA sequence was used (pYHSA2 vs. pYHSA3 in Table 2). Despite the fact that the total amount of HSA protein (intracellular+ extracelluar) from pYHSA3 was about 50% less than that from pYHSA2, Northern blot analysis revealed that the HSA mRNA levels from pYHSA3 were up to 50% higher than that from pYHSA2 (Fig. 4). The discrepancy observed with respect to the amount of HSA protein appeared to be derived from the instability of the Inu-HSA fusion protein as reported previously in the case where the MFα1 signal sequence from yeast was fused with the mature HSA protein [21].

**Effect of Host Strains on the Expression of HSA**

To compare the secretion levels of HSA among host strains with different genetic backgrounds, we tested three different *S. cerevisiae* strains (Table 3). Although the HSA secretion level of HSA was slightly higher in the strain L3262 than in the strain 2805 when the *GAPD* promoter was used for HSA expression, strain 2805 showed 25~50% higher levels of HSA expression as compared to strain L3262 when the *GAL10* promoter was used. Strain 2805 is a *pep4* disruptant strain, in which the levels of three major vacuolar proteases PrA (Pep4p), PrB, and PrC (CPY) are quite noticeably decreased [11], whereas the strain L3262 is a *PEP4* wild-type strain. It was reported that the expression of the vacuolar proteases is repressed in the presence of glucose [15]. Therefore, it could be possible that the marginal positive effect of *pep4* disruption in the strain 2805 was not observed in the glucose medium used for the expression from the *GAPD* promoter, while the positive effect of *pep4* disruption on the HSA expression was detected in the galactose containing medium for the activation of the *GAL* promoter.

The strain 334 is a *gal1* mutant, deficient in the enzyme that catalyzes the first step of galactose utilization. The strain also carries the *reg1-501* mutation, which eliminates glucose repression of *GAL* expression [10]. Therefore, the strain 334 can use galactose as a gratuitous inducer of the expression from *GAL* promoters in the presence of glucose as carbon source. In addition, it carries mutations in two protease genes, *pep4-3* and *prb1-1122*, which are useful for stabilizing overexpressed heterologous proteins. The total expression level of HSA from strain 334 appeared to be slightly lower when compared to the strain 2805 due to the relatively slower growth rate of the 334. However, considering that galactose is an expensive substrate for a large scale fermentation, strain 334 might have an advantage over the other strains as a host strain to produce large quantities of recombinant HSA.

**Table 3.** HSA expression in various *S. cerevisiae* strains.

| Plasmid | Promoter | Medium | Strain[a] | Yield (mg/l)[b] |
|---|---|---|---|---|
| pYHSA1 | GAPD | YPD | 2805 | 8 |
| | | | L3262 | 10 |
| pYHSA5 | GAL10 | YPDG | 2805 | 30 |
| | | | L3262 | 20 |
| | | | 334 | 20 |

[a]Host strains: *S. cerevisiae* 2805 (*MATα* pep4 ::HIS3 prb-Δ1.6R can1 his3-20 ura3-52), L3262 (*MATa ura3-52 leu2-3,112 his4-34*), and 334 (*MATα pep4-3 prb1-1122 ura3-52 leu2-3,112 reg1-501 gal1*). [b]The level of HSA in the culture supernatant of the transformants after 1-day cultivation was determined by densitometric tracing of 66-kDa of HSA band in Western blot.

**Secretory Expression of HSA in *S. cerevisiae***

Western blot analysis of the whole cell extracts and the culture supernatants from several yeast transformants showed that although the major form of HSA secreted into the culture supernatant was the mature form of 66 kDa, a smaller form of about 45 kDa was also present both in the cell extracts and in the culture supernatants. (Fig. 5A). The 66-kDa intact and the 45-kDa truncated HSA were mainly observed in the membrane fraction but were barely noticeable in the soluble cytoplasmic fraction (data not shown). Observation indicates that most HSA proteins synthesized in yeast were directed into the yeast secretion pathway and that the 45-kDa fragment was generated during the secretion process. In Western blot, the band corresponding to the 45-kDa fragment sometimes appears to be a doublet, reflecting the heterogeniety of the truncated products. It has been previously reported that the nature and amount of these truncated molecules varies according to the producer organism, growth regime, and secretion leader-sequence adopted [21].

To achieve a high-level secretory expression of HSA, we cultured the yeast transformant harboring pYHSA5, which showed the most efficient expression and secretion of HSA among the vectors tested, in shake-flasks for 3 days with galactose feeding. The time-course experiment for HSA production from the transformant (Fig. 5B) showed that the level of intact HSA in the culture supernatant reached up to 30 mg/l at 24 h of cultivation, which is comparable to the highest level of HSA previously reported to be produced in *S. cerevisiae* [16]. However, the level of HSA began to decrease progressively afterwards as the culture proceeded. As the intact 66-kDa HSA disappeared, the 45-kDa truncated products also disappeared in the culture supernatant,

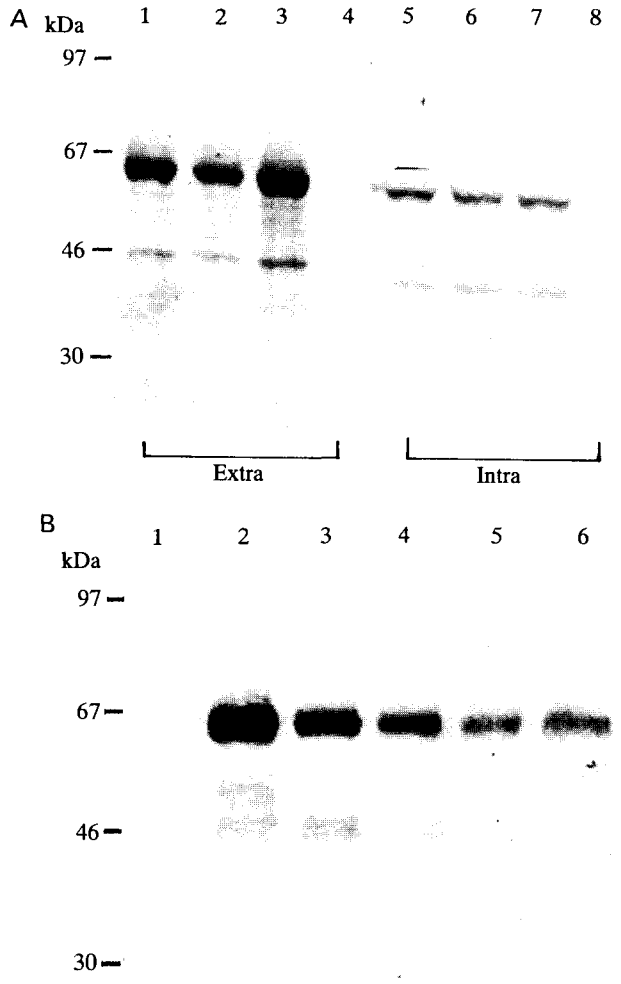


**Fig. 5.** Western blot analysis of HSA proteins expressed in *S. cerevisiae* transformants.

A, Expression and secretion of HSA in *S. cerevisiae*. Culture supernatant (lanes 1~4) and total cellular extract (lanes 5~8) were prepared from the *S. cerevisiae* L3262 transformants harboring pYHSA1 (lanes 1 and 5), pYHSA2 (lanes 2 and 6), pYHSA5 (lanes 3 and 7), and pGAPD-HIR (lanes 4 and 8), respectively, which were cultured in YPD or YPDG for 24 h. The samples were fractionated on 10% SDS-polyacrylamide gel, blotted on nitrocellulose membrane, and reacted with the HSA polyclonal antibody. Molecular weight standards are indicated on the left. B, Secretion kinetics of HSA in *S. cerevisiae*. The *S. cerevisiae* 2805 transformant harboring pYHSA5 was cultured in YPDG medium for 24 h and then fed with galactose at every 12 h up to 72 h in shake-flask cultivation to make the concentration of galactose at 2% in the culture medium. The culture supernatants (4 μl) taken at the indicated times were analyzed by Western blot as described in Panel A. Lanes 1 to 6, 4 μl of culture supernatants after 0, 24, 36, 48, 60, and 72 h growth in the shake-flask culture.

implying that all the HSA species were highly susceptible to proteolytic degradation in a prolonged culture. When the authentic HSA purified from human plasma was added to the culture of yeast cells, the disappearance of the authentic HSA in the culture supernatant was also observed as the culture proceeded (data not shown), supporting the presumption that the disappearance of HSA appears to be caused mainly by degradation in the culture supernatant. This observation also indicates that the degradation of recombinant HSA was not due to any alteration of the recombinant protein but derived from the inherent property of the protein.

In conclusion, we have designed and constructed several HSA expression vectors to achieve the high-level expression and secretion of HSA in the yeast *S. cerevisiae*. The recombinant HSA was highly expressed and efficiently secreted from the yeast but found to be very unstable in the prolonged culture supernatant. To overcome the problem of HSA degradation, efforts are currently being made through the combined approach of molecular genetics and culture optimization.

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