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Secretory Expression of Human α_{s1}-Casein in *Saccharomyces cerevisiae*

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Abstract A recombinant human α_{s1}-casein was expressed as a secretory product in the yeast *Saccharomyces cerevisiae*. Three different leader sequences derived from the mating factor α1 (MFα1), inulinase, and human α_{s1}-casein were used to direct the secretion of human α_{s1}-casein into the extracellular medium. Among the three leader sequences tested, the native leader sequence of human α_{s1}-casein was found to be the most efficient in the secretory expression of human α_{s1}-casein, which implies that the native leader sequence of human α_{s1}-casein might be used very efficiently for the secretory production of other heterologous proteins in yeast. The recombinant human α_{s1}-casein was proteolytically cleaved as the culture proceeded. Therefore, an attempt was made to produce human α_{s1}-casein using a *S. cerevisiae* mutant in which the *YAP3* gene encoding yeast aspartic protease 3 (YAP3) was disrupted. After 72 h of culture, most of the human α_{s1}-casein secreted by the wild type was cleaved, whereas more than 70% of the human α_{s1}-casein secreted by *yap3*-disruptant remained intact. The results suggest that YAP3 might be involved in the internal cleavage of human α_{s1}-casein expressed in yeast

Key words: Human α_{s1}-casein, secretion, leader sequence, proteolysis, *Saccharomyces cerevisiae*

Casein is a major milk protein constituting about 40% of the total protein content in the mature human milk [19]. In general, the ruminant casein consists of α-, β-, and κ-casein subunits. However, human casein has been thought to lack the α-casein which is a predominant fraction (~50%) of the bovine casein [19, 21]. Recently, the presence of α_{s1}-casein in human milk was revealed by the characterization of an α_{s1}-casein like protein purified directly from human milk [6, 23]. Subsequently, three types of human α_{s1}-casein mRNA transcripts have been cloned and sequenced [16].

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Since α_{s1}-casein accounts for only 0.06% of the total protein content of human milk [6], it seems almost impossible to supply a sufficient amount of native human α_{s1}-casein from human milk for the purpose of studying its structure and biological function. Therefore, we had previously overproduced human α_{s1}-casein up to 25% of the total cell protein via recombinant DNA technology using *E. coli* [18].

In the present work we have attempted to express human α_{s1}-casein as a secretory product. Since yeast is a more desirable host system for the production of human secretory proteins due to its similarity with animal cell secretion pathways, *S. cerevisiae* was used as the host. To investigate the effect of the leader sequence on the secretion of human α_{s1}-casein, the studies with three different leader sequences, the mating factor α1 (MFα1), the inulinase, and the human α_{s1}-casein leader, were carried out. In addition, we also examined the proteolysis of human α_{s1}-casein expressed in yeast as a secretory product. This is the first report on human α_{s1}-casein expression in yeast.

Materials and Methods

Strains and Transformation

The yeast strains used in this study are *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1 can1 his3*-200 *ura3*-52) [9], *S. cerevisiae* 334 (*MATα pep4*-3 *prb1*-1122 *ura3*-52 *leu2*-3, 112 *reg1*-501 *gal1*) [14] and *Saccharomyces cerevisiae* 334-HY (*MATα pep4*-3 *prb1*-1122 *ura3*-52 *leu2*-3, 112 *reg1*-501 *gal1 yap3::LEU2*) [17]. Yeast transformation was carried out by the lithium acetate method [15]. *Escherichia coli* DH5α was used for bacterial transformation and plasmid propagation [24].

Construction of Human α_{s1}-Casein Expression Vectors

Three different expression vectors, pYGMF-HαC, pYGINU-HαC, and pYGHαC-HαC, were constructed to express and secrete human α_{s1}-casein. The DNA fragment

encoding mature human α_{s1}-casein was amplified by PCR from the cloned human α_{s1}-casein cDNA using the following synthetic oligonucleotides: (1) 5'-CGC CGT CTA GAT AAA AGG AGG CCT AAA CTT CCT CTT-3' (nucleotide 74-91); (2) 5'-T ATT AAT TAC AAG AGA AGG CCT AAA CTT CCT CTT-3' (nucleotide 74-91); (3) 5'-G GAA TTC ATG AGG CTT CTC ATT CTC-3' (nucleotide 39-56); (4) 5'-A CGC GTC GAC TGT TCA CCA CTG TAG CAT GAC-3' (nucleotide 586-569). The 500 bp fragment encoding mature human α_{s1}-casein was amplified with the primers (1) and (4) and then was digested with restriction enzymes *Xba*I and *Sal*I. The plasmid pYGLP10 [10] was treated with *Bam*HI/*Xba*I, and the smallest fragment consisting of the *GAL10* promoter and MFα1 pre-pro leader signal sequence was eluted. Three fragments were ligated, resulting in the plasmid pYGMF-HαC. The 500 bp fragment encoding the mature human α_{s1}-casein was amplified with the primers (2) and (4), and then was digested by *Asn*I and *Sal*I. The INU1A signal sequence [10] was amplified by PCR using pYGILP10 [10] as a template with two following oligonuceleotides, 5'-CCG GAA TTC ATG AAG TTA GCA TAC-3' and 5'-TTT ATT AAT TCT CTT GTA ATT GAT-3'. The amplified 70 bp fragment was digested with *Eco*RI and *Asn*I. The two fragments were fused to the *Eco*RI/*Sal*I fragment of pYGLP10, resulting in the plasmid pYGINU-HαC. The cDNA encoding mature human α_{s1}-casein and its own signal sequence was amplified by the primers (3) and (4). The *Eco*RI/*Sal*I fragment of 600 bp was ligated with the *Eco*RI/*Sal*I fragment of pYGLP10, resulting in the plasmid pYGHαC-HαC.

Media and Culture Conditions

The YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) was used for the cultivation of host and yeast transformants. The YNBCAD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.5% casamino acids) was used for the selection of yeast transformants and also for the seed culture. For the induction of human α_{s1}-casein gene, the yeast transformants were grown in shake-flasks containing the YPDG medium (1% yeast extract, 2% bactopeptone, 1% glucose, and 1% galactose). Yeast extract, bactopeptone, yeast nitrogen base without amino acids, and casamino acids were purchased from Difco (U.S.A.).

Immunoblot Analysis of Human α_{s1}-Casein

After 1 ml of the culture broth was taken from the shake-flasks, the cells and extracellular medium were separated by centrifugation at 5000×g for 5 min. The extracellular fraction was precipitated by the addition of 100 µl of 100% trichloroacetic acid (TCA) and 100 µl of 0.1% deoxycholate (DOC). The precipitates were collected by centrifugation at 12,000×g for 10 min. The precipitates were washed with cold acetone and boiled for 5 min in 100 µl lysis buffer (50 mM Tris-HCl, 2% SDS, 100 mM NaCl, and 1 mM PMSF, pH 7.5). The resulting solution was subjected to 10% SDS-PAGE and immunoblotting. Immunoblotting was performed by the method of Towbin *et al.* [28]. The proteins were immunoblotted with the polyclonal antibody raised against the recombinant human α_{s1}-casein produced in *E. coli* [18].

RESULTS

Design and Construction of Human α_{s1}-Casein Secretion Vectors

Three different expression vectors were constructed to express and secrete human α_{s1}-casein in *S. cerevisiae*. They were designed to direct the secretion of human α_{s1}-casein by the aid of one of the following leader sequences, the MFα1 pre-pro leader, the inulinase (INU1A) leader, and the native leader sequence of human α_{s1}-casein, and named pYGMF-HαC, pYGINU-HαC and pYGHαC-HαC, respectively (Fig. 1B). The transcriptions of the fused genes in all the plasmids were designed to be regulated by *GAL10* promoter and *GAL7* transcription terminator (Fig. 1A). In cases of MFα1 pre-pro leader- and inulinase leader-directed secretion, the carboxy-terminal dibasic residue, Lys-Arg, of the leader peptides is expected to be processed by the action of KEX2 endoproteinase. On the other hand, the native leader peptide of human α_{s1}-casein is expected to be processed by the signal peptidase which is not identified as of this time.

Expression and Secretion of Human α_{s1}-Casein in *S. cerevisiae*

S. cerevisiae 2805 was transformed with each of the plasmids pYGMF-HαC, pYGINU-HαC, and pYGHαC-HαC, and the resulting transformants were cultured in YPD and YPDG media. At first, we attempted to analyze human α_{s1}-casein by SDS-PAGE but failed to directly detect it on the gel, indicating that a very small amount of or no human α_{s1} casein was secreted extracellularly. After 48 h of culture, the culture supernatants were analyzed by immunoblot (Fig. 2). None of the induced proteins were detected in the cultures of non-transformants in YPD and YPDG media (Fig. 2, lanes 1 and 2) and also in the cultures of transformants in YPD medium (Fig. 2, lanes 3, 5, and 7). On the other hand, two discrete proteins with molecular weights of ca. 20 and 27 kDa were detected in YPDG cultures of the transformants (Fig. 2, lanes 4, 6, and 8). The 27 kDa protein (Fig. 2, band i) appears to be intact human α_{s1}-casein because human α_{s1}-caseins, which were isolated from the mature milk [6, 23] and was produced in *E. coli* [18], showed the same molecular weight on SDS-PAGE. However, the theoretical molecular weight of

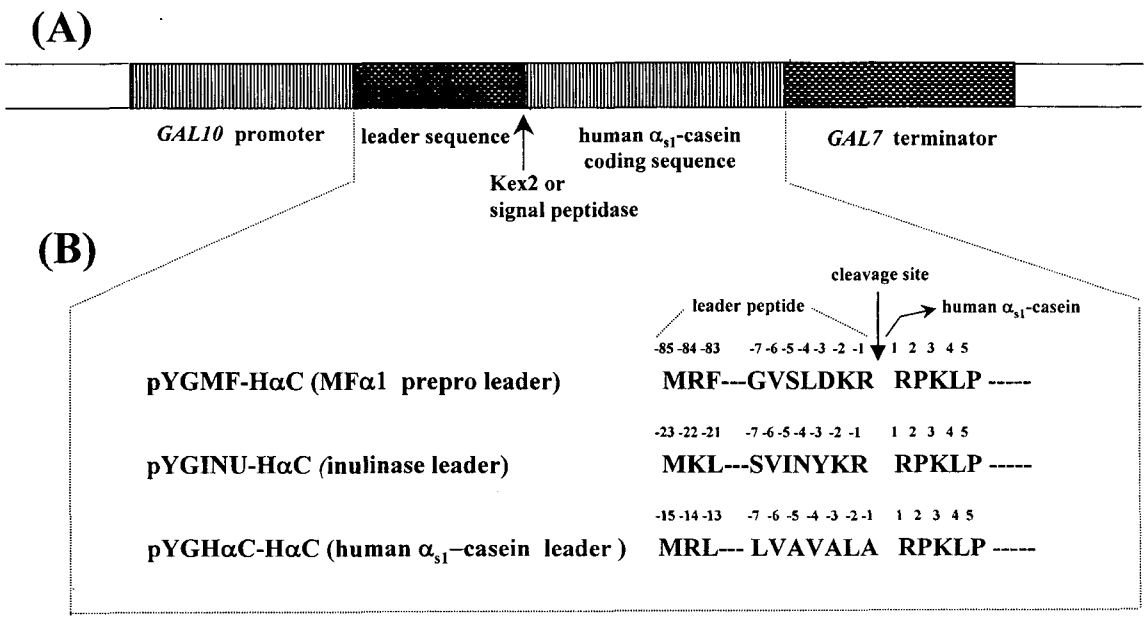

Fig. 1. (A) Basic structure of the plasmid used in this study and (B) amino acid sequences representing the junction regions between three different leader peptides and human α_{s1}-casein.

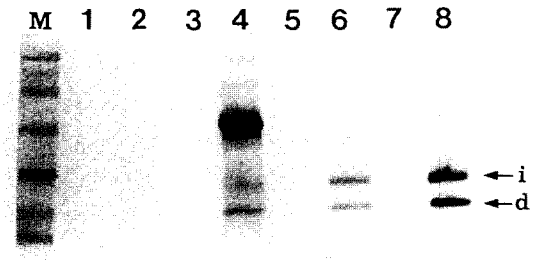

Fig. 2. Immunoblot analysis of the recombinant human α_{s1}-casein secreted by untransformed (lanes 1, 2) and transformed (lanes 3-8) *S. cerevisiae* 2805.

The secretion of human α_{s1}-casein was directed by three different leader sequences. The cells were grown in YPD (odd-numbered lanes) and YPDG (even-numbered lanes) media for 48 h. Lanes: M, prestained protein molecular weight marker (97, 67, 43, 30, 20.1, 14.4 kDa); 1, 2, untransformed strain; 3, 4, MFα1 pre-pro leader; 5, 6, inulinase leader; 7, 8, human α_{s1}-casein leader. Bands: i, intact human α_{s1}-casein; d, cleaved human α_{s1}-casein.

human α_{s1}-casein estimated from the amino acid sequence is ca. 20 kDa. It is not yet clear why the human α_{s1}-casein migrates aberrantly by SDS-PAGE as 27 kDa proteins. Recently, the aberrant electrophoretic mobility of human parathyroid hormone (1-80) secreted in *S. cerevisiae* has been reported [29], where the 9 kDa protein migrated as 14 kDa proteins on SDS-PAGE. It is presumed that such an aberrant electrophoretic mobility can be caused by the conformational difference between proteins with the similar molecular weight. Therefore, the 20 kDa protein (Fig. 2, band d) seems to be a degraded product of the human α_{s1}-casein generated by an endoproteolytic activity. When the secretion was directed by the MFα1 pre-pro leader sequence, the protein with a molecular weight of ca. 43 kDa was induced and secreted in the YPDG medium (Fig. 2, lane 4). This appears to be the recombinant human α_{s1}-casein fused with the MFα1 pre-pro region, resulting from the incomplete KEX2 processing. Since the purified human α_{s1}-casein sufficient for further characterization could not be obtained due to low secretion level, it is not clear yet whether correct processing by a signal peptidase took place at the junction between intact human α_{s1}-casein and its own signal peptide. However, it seems that the native leader peptide of human α_{s1}-casein was correctly processed during the secretion in yeast because all of the human α_{s1}-casein proteins secreted by three different leader peptides comigrated on SDS-PAGE (Fig. 2, lanes 4, 6, and 8).

The native leader sequence of human α_{s1}-casein was found to be the most efficient of the three different leader sequences used in the secretory expression of human α_{s1}-casein from *S. cerevisiae*. This result implies that the native leader sequence of human α_{s1}-casein might be used most efficiently in the secretory production of a variety of heterologous proteins in yeast.

Expression and Secretion of Human α_{s1}-Casein in a *yap3*-Disrupted Yeast Mutant

To investigate whether the yeast aspartic protease 3 (YAP3), an endoprotease residing in the plasma membrane, is involved in the proteolytic cleavage of human α_{s1}-casein, the plasmid pYGHαC-HαC into *S. cerevisiae* 334 and *S. cerevisiae* 334-HY was transformed. The latter is a mutant strain in which the *yap3* gene encoding yeast aspartic protease 3 is disrupted [17]. When the transformants

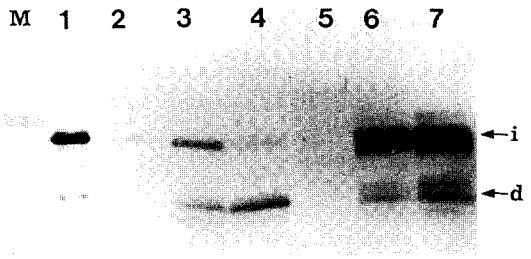

Fig. 3. Proteolytic cleavage of human α_{s1}-casein secreted by the transformed wild-type (lanes 2-4) strain and *yap3* disruptant (lanes 5-7).

Lanes: M, prestained protein molecular weight marker (30.6 and 17.8 kDa); 1, the *E. coli* derived and purified recombinant His-tag human α_{s1}-casein protein; 2, 5, after 24-h of culture; 3, 6, after 48-h of culture; 4, 7, after 72-h of culture. Bands: i, intact human α_{s1}-casein; d, cleaved human α_{s1}-casein.

of wild-type and *yap3* disruptant were cultured in the YPDG medium, the transformed wild-type showed an increase in the proteolytically cleaved human α_{s1}-casein with an increasing culture time (Fig. 3, lanes 2-4). As already noted, the molecular weight of Met-human α_{s1}-casein with a 6×His tag in the C-terminus produced by *E. coli* is ca. 21 kDa but migrated as 28 kDa proteins on SDS-PAGE (Fig. 3, lane 1). After 72 h of culture, more than 70% of the human α_{s1}-casein secreted by the *yap3* disruptant remained intact, whereas most of the human α_{s1}-casein secreted by the wild-type strain was cleaved. These results confirm again that 20 kDa protein is an endoproteolytic product of the human α_{s1}-casein. These results also indicate that YAP3 is one of the endoproteases involved in the internal cleavage of human α_{s1}-casein secreted by *S. cerevisiae*.

DISCUSSION

In addition to the main biological function of caseins as a source of amino acids and a vehicle for phosphorous and calcium ion adsorption, caseins are known to have very interesting functions to provide biologically active peptides. A variety of small peptides derived from caseins have been reported to exhibit physiologically important activities such as opiate, antihypertensive, antithrombotic, immunomodulatory, and calcium absorption enhancing activities [4, 11, 12, 26]. Furthermore, caseins are very interesting in a view of molecular evolution because they are one of the most rapidly evolving families of proteins [22, 30]. Therefore, it is important to study their structure and biological functions, where the supply of casein protein is required. In this work, we attempted to develop the secretory expression system of human α_{s1}-casein in *S. cerevisiae* in an effort to provide it in large quantities.

Protein secretion from yeast is usually directed by a leader sequence which is fused with the N-terminal of mature protein, and the leader sequence influences the quantity and quality of the secreted protein. So far, two approaches have been used to secrete heterologous proteins from yeast: the use of a heterologous (mammalian or nonyeast) leader sequence; and the use of a homologous (yeast) leader sequence. Although there have been some reports on notable successes in the protein secretion with heterologous leader sequence [27], in many cases, its use in yeast has resulted in a very low to no secretion efficiency [5, 13]. In the present study, however, it was demonstrated that human α_{s1}-casein can be efficiently secreted in *S. cerevisiae* using its native leader sequence, offering the potential use of human α_{s1}-casein leader sequence in the secretory production of other various heterologous proteins in yeast.

YAP3 is a proprotein convertase that cleaves at single and paired basic residues [1, 2, 20]. The purified YAP3 protease is known to be capable of processing a number of prohormones such as mouse pro-opiomelanocortin and its fragments, human adrenocorticotropic hormone, bovine proinsulin, porcine cholecystokinin, dynorphin, and amidorphin [3, 7, 8]. More recently, it was found that the internal cleavage of human parathyroid hormone could be reduced very efficiently using the same *yap3* disruptant used in this study [17]. Looking into the amino acid sequence of human α_{s1}-casein, human α_{s1}-casein contains paired dibasic residues (-Arg^{109}-Arg^{110}-) which can be recognized by YAP3. Therefore, an attempt to reduce the proteolysis of human α_{s1}-casein by using the *yap3* disruptant as a recombinant host was made. As a consequence, the aberrant proteolytic cleavage of human α_{s1}-casein was considerably reduced by the use of this mutant strain.

According to the densitometric scanning of the induced proteins using the *E. coli* derived and purified Met-human α_{s1}-casein with a His-affinity tag at the C-terminus [18], ca. 0.6 mg/l of the intact human α_{s1}-casein was expressed as a secretory product using a *yap3*-disrupted mutant of *S. cerevisiae*. This accounted for ca. 3.8% of the total extracellular protein, which is much higher than the content in human milk. However, this expression level was not high enough to obtain a sufficient amount of the purified human α_{s1}-casein from the yeast culture medium. Therefore, further attempts will be made to increase the expression level of human α_{s1}-casein in *S. cerevisiae*. In addition, the studies on the characterization of recombinant human α_{s1}-casein will be carried out in order to understand the structure and function of the mature human α_{s1}-casein.

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