Short Communication

Effects of signal sequences on the secretion of hen lysozyme by yeast: construction of four secretion cassette vectors

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Introduction

Recombinant protein expression is important in many fields—medicine, biochemistry, molecular biology, etc.—as well as in industry. In general, bacterial, fungal, animal and plant cells have been used for this purpose. In the case of the bacterium *Escherichia coli*, recombinant proteins often accumulate as inclusion bodies, although expression level is high (Imoto et al., 1997). Therefore, the inclusion body must be renatured into active forms. Moreover, in many cases, methionine is not removed from the N-terminal end of the protein, which may affect the function or structure of the protein (Mine et al., 1997). On the other hand, as is the case for animal and plant cells, the level of expression is generally low (Haynes and Weissmann, 1983; Kaufman et al., 1987; Miyaji et al., 1990). Fungi, especially the yeast *Saccharomyces cerevisiae*, can secrete foreign protein in an active form and the secretion level is higher relative to that of animal or plant cells. Glycosylation occurs both in yeast as well as in animal cells (Ballou, 1990). Thus, expression in yeast is one of the best ways to acquire and purify large quantities of protein in an active form.

Expression and secretion of various mammalian proteins in yeast have been reported: interferon α (Hitzeman et al., 1981), interferon γ (Derynck et al., 1983), interleukin 2 (Shaw et al., 1985) and epidermal growth factor (Brake et al., 1984), Fab (Horwitz et al., 1988). However, recombinant proteins are not always secreted from yeast, which is dependent on the target protein, host strain and secretion—signal sequence. The choice of a suitable secretion—signal sequence for a given protein is an important factor in the final yield of secreted protein.

The addition of a secretion—signal sequence onto the mature protein is essential to acquire an active protein. One way to select the signal sequence is to use the original signal sequence of the desired protein gene (Jigami et al., 1986; Nakamura et al., 1986). However, it is limited only to secretory proteins. Alternatively, a yeast signal sequence may be used. In this case, it can be applied not only to secretory proteins but also to non-secretory proteins.

Before a foreign protein is expressed in yeast, some trials are required to find out which signal sequence is most suitable. We constructed four ‘secretion cassette vectors’ containing four different yeast secretion signals: αF (Brake, 1990; Hitzeman et al., 1990), KILM1 (Skipper et al., 1985), PHO1 (Laroche et al., 1994) and SUC2 (Hitzeman et al., 1990). These signal sequences originate from the yeast secretory proteins alpha factor 1 peptide hormone, killer toxin type 1, acid phosphatase and invertase, respectively. To date, there are few reports that quantitatively evaluate the efficiency of different signal sequences on the secretion of a single protein. Here the efficiency of secretion of hen lysozyme was examined.

Materials and methods

Strains, plasmids and phages

*Saccharomyces cerevisiae* strain AH22 (Miyanohara et al., 1983), which was kindly supplied by Dr F. Hamada (Chemotherapeutic Research Institute, Kumamoto, Japan), was used for the expression and secretion of hen lysozyme. Yeast cells were grown in a modified Burkholder minimal medium (Hashimoto et al., 1996). pHA394 has a streptomycin resistant gene between the *Sac* and *BamH* sites downstream from the PHO5 promoter. M13 phage vector pHA474 has the sequence *Sac*-[*SUC2 signal sequence*]-*EcoRI*-HindIII*-SpHl*-PstI*-XhoI*-BamHI*-XhoI instead of the multi-cloning region of M13 mp19.

Construction of ‘secretion cassette vector’

M13 phage vector pHA474 was treated with *PstI*, T4 DNA polymerase and *XhoI* in series. On the other hand, pUC119 was treated with *EcoRI*, DNA polymerase I Klengow fragment and *Sac* in series. These digests were ligated together. The nucleotide sequence, *Sac*-*[SUC2 signal sequence]*-*EcoRI*-HindIII*-SpHl*-SacI*-KpnI*-SmaI*-BamHI*-XhoI, was confirmed by sequencing, yielding pNKsUC (Figure 1). DNA fragments containing the αF, KILM1 and PHO1 signal sequences were amplified by PCR, in which *Sac* and *EcoR* sites were introduced at the 5’- and 3’-ends, respectively. These fragments were digested with both *Sac* and *EcoR* sites and ligated between the *Sac* and *EcoR* sites in pNKsUC, yielding pNKαF, pNKαKIL and pNKPH (Figure 1).

Construction of lysozyme expression vectors

The lysozyme gene amplified by PCR was digested with both *EcoRI* and *BamH*, and ligated to pNKαF, pNKαKIL and pNKPH and pNKsUC. Mutagenesis was performed by the Kunkel method for fusing the lysozyme gene to the signal sequence (Kunkel et al., 1987). Oligonucleotides used in mutagenesis were 5’-CTCGAGAAAGAAAAAGTCTTTTGGAGA3’- 5’-CCAATTACCTCGTAAAGTCTTTGGA-3’ and 5’-AAGATATCTGCAAAAGTCTTTTGGAGA3’- 5’-CTGCCTCTGCCCTAAGTCTTTTGGAGA3’ for pNKαF, pNKαKIL, pNKPH and pNKsUC, respectively. The fusions were digested with both *Sac* and *BamH*, and ligated into the expression vector pHA394.

Yeast transformation, cultivation of yeast and purification of recombinant lysozymes

Yeast AH22 was transformed with pHA394 derivatives (Hinnen et al., 1978). Cultivation of yeast and purification of lysozymes were carried out as previously described (Hashimoto et al., 1996).
Fig. 1. (A) The structure of ‘secretion cassette vector’. Four ‘secretion cassette vectors’ containing αF, KILM1, PHO1 and SUC2 signal sequences are named pNKαF, pNK1, pNKPHO and pNKSUC, respectively. lacP/O means both lac promoter and operator. (B) The nucleotide sequences of αF, KILM1, PHO1 and SUC2 secretion signal. Amino acids estimated from the nucleotide sequences are represented in one letter code.

Results

Productions and purifications of recombinant lysozymes

The lysozyme gene was inserted into pNKαF, pNK1, pNKPHO and pNKSUC. Each signal sequence was fused to the lysozyme gene directly by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). The fusion gene was inserted into the SalI and BamHI sites of the yeast expression vector pHA394. Expression and production by yeast was performed as previously described (Hashimoto et al., 1996). Cation-exchange chromatograms of each product are shown in Figure 2. The fractions, a-3, b-2, c-1 and d-1, showed activity levels similar to that of wild-type (Table I). As for the αF and KILM1 signal sequences, sub-products (a-1, a-2 and b-1) were observed. The lysozyme activity was retained in a-2 and b-1, and these were lysozyme derivatives (Table I). Purity and molecular weight of secreted proteins were examined using TOF/MS spectrometry (Table I). All except for the fraction a-1 could be thus characterized. SDS-PAGE, estimated the molecular weight of the fraction a-1 as higher than 210 kDa (data not shown). Perhaps a-1 was the lysozyme where the glycosylated signal sequence peptide was added. As for the proteins in a-3 and b-2 and the main peaks of PHO1 and SUC2, the molecular weights were the same as that of wild-type lysozyme. The molecular weight of the fraction a-2 was 15 466 Da, which was higher than that of wild-type. The molecular weight of the fraction b-1 was 16 130 Da.

Table I. Mass, N-terminal sequence and yield of secreted lysozyme from yeast

<table>
<thead>
<tr>
<th>Signal</th>
<th>Fraction</th>
<th>Mass (Da)</th>
<th>N-terminal Sequence</th>
<th>Yield (%)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td></td>
<td>14316</td>
<td>Lys-Val-Phe</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>αF</td>
<td>a-1</td>
<td>N.D.</td>
<td></td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>a-2</td>
<td>15466</td>
<td></td>
<td>0.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>a-3</td>
<td>14313</td>
<td>Lys-Val-Phe</td>
<td>13.0</td>
<td>100</td>
</tr>
<tr>
<td>KILM1</td>
<td>b-1</td>
<td>16130</td>
<td>Leu-Asn-Asp</td>
<td>2.6</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>b-2</td>
<td>14313</td>
<td>Lys-Val-Phe</td>
<td>1.2</td>
<td>100</td>
</tr>
<tr>
<td>PHO1</td>
<td>–</td>
<td>14316</td>
<td>Lys-Val-Phe</td>
<td>2.1</td>
<td>100</td>
</tr>
<tr>
<td>SUC2</td>
<td>–</td>
<td>14316</td>
<td>Lys-Val-Phe</td>
<td>2.0</td>
<td>100</td>
</tr>
</tbody>
</table>

aFractions are shown in Figure 2.
bMolecular weights of recombinant proteins were measured with MALDI-TOF/MS (Voyager).
These yields are per liter after cultivation for 120 h.
These are specific lysis activities to native lysozyme (Verhamme et al., 1988). Standard deviations were <10%.
N.D., not determinable.
N-terminal sequences of secreted lysozymes

N-terminal sequences of secreted lysozymes were analyzed for three residues by protein sequencing (Table I). The sequence of fractions a-3, b-2, c-1 and d-1 was that of mature lysozyme, Lys-Val-Phe, while we were unable to determine sequence for fractions a-1 and a-2, perhaps because of N-terminal blocking. That of the fraction b-1 gave Leu-Asn-Asp, which exists in the KILM1 signal. This was consistent with the molecular weight measurement.

Discussion

Hen lysozyme gene was introduced into four signal cassette vectors and lysozyme was secreted by yeast. The lysozyme was correctly processed in all cases. The relative secretion levels were as follows: αF > KILM1 > PHO1 and SUC2. In the case of PHO1 and SUC2, correctly processed proteins were obtained (Figure 2). The fusion of the SUC2 signal sequence and interferon-α2, Met-interferon-α2 or human serum albumin is all correctly processed (Hitzeman et al., 1990). The right side of the signal cleavage site seems to be tolerant of varied amino acids. PHO1 signal is successfully applied to tick anticoagulant peptide (TAP) in the yeast Pichia pastoris (Laroche et al., 1994). As for αF and KILM1 signal sequences, both correctly and incorrectly processed sub-products are observed. Skipper et al. (1985) applied the KILM1 signal to a cellulase gene. KILM1 signal used in this study has the prepro-structure; Met1-Ala26 and Leu27-Arg44 are pre- and pro-sequences, respectively (Tipper and Bostian, 1984). Mis-processed product was mainly that cleaved between Ala26 and Leu27 in the signal. If the lysosome gene was linked after Ala26 in the KILM1 signal, the increase in the secretion level of sub-products misprocessed increased as the cultivation lengthened. These results shows that lysozyme correctly processed can be obtained in high purity by short cultivation times (data not shown).

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References