Note

Secretion of Hen Egg White Lysozyme from Klyuyveromyces lactis

Ryoichi Tanaka, Matsujiro Ishibashi, Hiroko Tokunaga, and Masao Tokunaga

Laboratory of Applied and Molecular Microbiology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan

Received June 9, 2000; Accepted August 11, 2000

Hen egg white (HEW) lysozyme was correctly processed and efficiently secreted from an alternative yeast, Klyuyveromyces lactis. We constructed secretion vectors using PHO5, PGK, and LAC4 promoters, and found that the highest secretion was obtained under the direction of the PGK promoter in non-selective rich medium. K. lactis secreted HEW lysozyme with twofold higher efficiency than S. cerevisiae, estimated by using a K. lactis-S. cerevisiae shuttle vector.

Key words: lysozyme; secretion; Klyuyveromyces lactis; shuttle vector

Among various host-vector systems, yeast is a very useful host cell for the extracellular production of recombinant gene products, since it is a safe eukaryotic microorganism with well-established fermentation technology. Recently, non-Saccharomyces (or alternative) yeasts, such as Klyuyveromyces lactis and Pichia pastoris, have been used as new host cells.1)

Lysozyme, which catalyzes the hydrolysis of the β-(1,4)-glycosidic linkage between N-acetylglucosamine and muramic acid of the bacterial peptidoglycan, is one of the best characterized and used enzymes.2) It is included in a cold medicine and eye drops as an anti-inflammation agent as well as a food preservative as a bactericide. Recently, a novel antimicrobial mechanism of lysozyme independent of its catalytic function has been described against both Gram-negative and Gram-positive bacteria,3) and improvement of bactericidal action was reported using protein engineered lysozyme molecules produced by Saccharomyces cerevisiae.4)

We have been studying efficient secretion systems for foreign gene products in S. cerevisiae.5,6) Schizosaccharomyces pombe,7) and K. lactis,8) and found that K. lactis is a more suitable host cell for the recombinant production of mouse α-amylase than S. cerevisiae. To establish a highly efficient secretion system for recombinant hen egg white (HEW) lysozyme, here, we attempted to construct various K. lactis secretion vectors, and directly compared its secretion efficiency to that of S. cerevisiae using K. lactis-S. cerevisiae-E. coli shuttle vector.

We used PHO5 and PGK promoters from S. cerevisiae, and an inducible K. lactis LAC4 promoter for the construction of secretion vectors. The HEW lysozyme gene including its own secretion signal was isolated from M13 vector Ps-Lz7369) and cloned into K. lactis expression vectors, pEPHO and pEPGK410) to construct pHFl-HeLz and pPG-HeLz, respectively (Fig. 1). The Smal site of pEPHO was changed to Xhol with insertion of an Xhol linker, and EcoRI/

---

1) To whom correspondence should be addressed. Masao Tokunaga, FAX:81-99-285-8634; E-mail: tokunaga@chem.agri.kagoshima-u.ac.jp

Abbreviations: HEW, hen egg white; PAGE, polyacrylamide gel electrophoresis
SalI-digested HEW lysozyme gene was inserted at EcoRI/XhoI sites of modified pEPHO to obtain pPH-HeLz. The end-filled HEW lysozyme gene was ligated at EcoRI-digested pEPGK41 after end-filling treatment to construct pPG-HeLz. The PGK promoter region of pPG-HeLz (SalI/KpnI fragment) was replaced by the K. lactis LAC4 promoter(11) to obtain pLA-HeLz (Fig. 1). To compare the secretion efficiency of lysozymes from two yeast strains, K. lactis and S. cerevisiae, the lysozyme expression cassette, PGK promoter-lysozyme-PGK terminator (SalI/BamHI fragment) from pPG-HeLz was cloned into the SalI/BamHI-digested K. lactis-S. cerevisiae shuttle vector pSK1(10) to construct pSV-HeLz (Fig. 1). For the yeast host cells, K. lactis MD2/1 (MATa, uraA, argA1, lysA1, ral1, rag2, cir-) and PM6-7A (MATa, ura4, ade2, cir-) were used. In addition, for the pSV-HeLz, S. cerevisiae YNN27 (MATa, trp1, ura3, cir-) and S150-2B (MATa, his3, leu2, trp1, ura3, cir-) were used. Transformants were selected and maintained on complete synthetic medium(12) (CSM, 0.67% yeast nitrogen base and amino acid mixture) minus uracil/2% glucose plates. For the lysozyme secretion, 1.0 ml of seed culture in CSM (minus uracil)/glucose was inoculated in 5 ml of CSM (minus uracil)/glucose, CSML (lactose instead of glucose), YPD (1% Bacto yeast extract, 2% Bacto peptone, and 2% glucose), or YLP (lactose instead of glucose of YPD) media in test tubes (1.3 × 15 cm), and cells were grown for 4 days at 30°C with shaking. Secretion of lysozyme reached the maximum level after 4-5 days culture in all transformants.

The lysozyme activity was assayed as follows: 0.5 ml of Micrococcus lysodeikticus cell suspension (Sigma M3770, 1 mg of freeze-dried cells in 4 ml of 0.1 M acetate buffer, pH 6.0) was incubated with 0.5 ml culture supernatant (adjusted to pH 6.0 with acetate buffer) at 30°C for 10 min, and the decreased OD660 was measured as cell lytic activity. One unit was defined as 0.01 OD decrease at 450 nm for 1 min. The results are summarized in Table 1. All experiments were done at least three times and the average value is shown. The maximal amount of lysozyme, 15.5 units, was secreted from MD2/1(pPG-HeLz) in non-selective YPD medium. For an unknown reason, lysozyme was not produced from MD2/1 cells in synthetic CSM medium, although it can grow normally in CSM. In contrast, lysozyme was secreted from PM6-7A cells to almost the same extent both in CSM and YPD. Around 10 units of lysozyme was inductively secreted from MD2/1(pLA-HeLz) in YP-lactose and from PM6-7A(pLA-HeLz) in CSM-lactose, indicating that the LAC4 promoter vector can function efficiently irrespective of K. lactis strains. It was confirmed that the secretion under the direction of LAC4 promoter was inducible by lactose as shown in Table 1. We examined the plasmid stability during culture in YPD for 4 days. pPH-HeLz and pPG-HeLz were maintained with 20.3-23.4% efficiency in both K. lactis host cells, while pLA-HeLz was with 40-45%, suggesting that the expression of lysozyme negatively affected plasmid stability.

We purified lysozyme from the culture supernatant of PM6-7A(pPG-HeLz). One liter of culture supernatant, of which the pH was adjusted to 7.5, was put on a HiPrep CM Sepharose FF column (Pharmacia) at 1 ml/min, and the column was eluted in Tris-HCl buffer, pH 7.5, with a linear gradient of 0-0.5 M NaCl after washing with 2 bed volumes of Tris-HCl buffer, pH 7.5. The lysozyme fraction eluted at 0.35 M NaCl was apparently homogeneous (Fig. 2) by SDS-polyacrylamide gel electrophoresis (PAGE) and the band was stained with anti-HEW lysozyme antiserum (Chemicon). The mobility of secreted lysozyme on SDS-PAGE was identical to that of authentic HEW lysozyme (Sigma L6876). The NH2-terminal amino acid sequence of this band, after bloting onto a poly-vinyliden difluoride membrane, was found to be KVFGRXELAA (X is unknown), demonstrating that the HEW lysozyme precursor was correctly processed and secreted from K. lactis. The yield of purified HEW lysozyme is ~0.5 mg/liter culture, which is about twice more than that from S. cerevisiae(19).

Table 1. Lysozyme Activities Secreted from K. lactis Transformants Using Various Promoters for Expression Vectors, and Different Media Conditions for Growth

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Activity (Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MD2/1</td>
</tr>
<tr>
<td></td>
<td>CSM/CSML YPD YPL</td>
</tr>
<tr>
<td>pPH-HeLz</td>
<td>0  1.6  3.0  2.3</td>
</tr>
<tr>
<td>pPG-HeLz</td>
<td>0  15.5  3.2  3.6</td>
</tr>
<tr>
<td>pLA-HeLz</td>
<td>0  0.5  10.2  0.3</td>
</tr>
</tbody>
</table>

Fig. 2. SDS-polyacrylamide Gel Electrophoretogram of Purified HEW Lysozyme Secreted from K. lactis.

A. SDS-PAGE (14% gel) with Coomassie blue staining. B. Western blotting with anti-HEW lysozyme antiserum. Lane 1, molecular weight standards (A, Bio-Rad Low molecular weight, B, prestained marker(New England BioLabs.)); lane 2, authentic HEW lysozyme; lane 3, purified HEW lysozyme secreted from K. lactis. Lz, lysozyme.
Using the K. lactis-S. cerevisiae shuttle vector containing both origins of a 2-μm plasmid from S. cerevisiae and of pKD1 from Kluyveromyces, the efficiency of lysozyme secretion from S. cerevisiae and K. lactis was directly compared. As shown in Fig. 3, K. lactis MD2/1(pSV-HeLz) secreted 8.6 U of lysozyme, which is twice the amount of that from the S. cerevisiae YNN27 transformant. The stability of pSV-HeLz in YPD medium was 30–33.8% in K. lactis and 60–70% in S. cerevisiae. K. lactis still secreted more lysozyme despite its lower capacity for plasmid distribution than S. cerevisiae. Thus, the increased plasmid stability e.g. application of a centromere plasmid10 or chromosome integration of the lysozyme gene, will much improve the secretion efficiency. During this study, Maullu et al. reported the high level production of human lysozyme from K. lactis grown in cottage cheese whey.13)

In conclusion, we succeeded in extra-cellular production of HEW lysozyme from K. lactis, and found that K. lactis is a more suitable yeast host than S. cerevisiae for HEW lysozyme production.

Acknowledgment

We are grateful to Drs. Hiroshi Fukuhara, and Hisham Ibrahim for the gift of strains and plasmids.

References