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Analysis of Secretion Behavior of Human Lysozyme from Recombinant *Saccharomyces cerevisiae*

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Abstract Effects of signal sequences, protein sizes and dissolved oxygen on the secretion of human lysozyme from a recombinant yeast were experimentally characterized. The systems consisted of *Saccharomyces cerevisiae* host SEY2102 that was transformed with two different plasmids. These plasmids were identical with an exception to the plasmid pMC614, which contained the native yeast MFα1 sequence and the plasmid pMC632 with the non-native rat α-amylase signal sequence. The expression of human lysozyme was controlled by the *ADH*I promoter. The native yeast *MF*α1 signal sequence was more efficient than the non-native rat α-amylase signal sequence in directing the secretion of human lysozyme. Lysozyme secreted with the α-amylase signal was retained inside the cells and released to the medium very slowly, thereby causing a lower cell growth rate and a decreased product secretion rate. Lysozyme was secreted more efficiently than invertase, which is an order of magnitude bigger in molecular size compared to lysozyme, which was under the direction of the *MF*α1 signal sequence, suggesting that protein sizes may affect the secretion efficiency. When expressed in anaerobic conditions in the medium where the *ADH*I promoter was derepressed, the amount of lysozyme secreted was about twice higher than that of the aerobic culture. However, the secretion rates were identical. This result showed that the dissolved oxygen level may affect the efficiency of protein secretion only, and not the secretion rate of the product protein.

Key words: *Saccharomyces cerevisiae*, human lysozyme, secretion

The secretion of heterologous proteins from recombinant microorganisms has emerged as an important process that has a potential to greatly reduce separation costs in industrial fermentations [11]. Secretion can also eliminate many problems associated with overproduction of recombinant proteins in microbial hosts. These problems may include the formation of inclusion bodies that require unfolding and refolding processes for biological activity and the degradation of product proteins by cellular proteases. The baker's yeast, *S. cerevisiae*, is an attractive host for the expression of recombinant proteins because it does not possess endotoxins and, more importantly, the Food and Drug Administration (FDA) has classified it as "generally recognized as safe" (GRAS). The *S. cerevisiae* secretion pathway has been studied extensively and reviews have been offered [2, 17]. A high-cell fermentation method has also been demonstrated for *S. cerevisiae* using a fed-batch strategy [8]. Proteins targeted for secretion contain a short, hydrophobic, amino-terminal, along with the peptide sequence and they are usually composed of about 20 different amino acids. This signal sequence is responsible for the concomitant translation-translocation of secreted proteins which were added into the lumen of the endoplasmic reticulum [6].

Lysozyme is a hydrolytic enzyme (E.C, 3.2.1.17) that cleaves the α1→4 glycosidic linkages in the murein cell wall of various bacteria. Lysozyme is found in different body fluids and has been widely studied in the past. As a result, its three-dimensional structure, mechanism of lytic activity, and biochemistry have been determined by using tools such as x-ray crystallography and magnetic resonance [1, 5, 16]. The human lysozyme gene (HLZ) product containing 130 amino acids acquires a mass of approximately 14.6 kDa, and is cross-linked by four disulfide bonds [3]. These cross-links assist to mainly stabilize the enzyme and lead to correct folding and biological activity. Lysozyme has also been used commercially in some countries as a food and pharmaceutical preservative. In a clinical application, lysozyme is used in conjunction with antibiotics for the synergistic effect in the treatment of some diseases.

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In the present experiment, the human lysozyme structural gene (HLZ) was expressed under the control of a native yeast *ADH*I promoter. Plasmid pMC614 secrets its product with an aid of the native yeast *MF*α1 signal sequence, while plasmid pMC632 employs the rat α-amylase signal sequence [3]. *ADH*I is the promoter of yeast alcohol dehydrogenase which is a major enzyme in an alcoholic fermentation process that converts acetaldehyde to ethanol [4]. There are several reasons as to why these systems were chosen. First, lysozyme in each of the plasmids is secreted under direction of the same promoter with the aid of two different signal sequences. It was intended to compare the secretion rate and its efficiency rate between the native yeast signal sequence and the non-native signal sequence. In addition, at different protein expression rates, by changing the culture conditions with the dissolved oxygen, protein secretion rates were closely measured. Second, human lysozyme is a relatively small protein and is not glycosylated. It was considered that its secretion behavior could offer an interesting comparison to our previous study conducted with invertase [10]. Third, lysozyme is enzymatically active and, therefore, it is possible to quantitate its amount by using enzymatic assay methods. In addition, the lysozyme used here is from a human source with a potential to be used commercially as a food and pharmaceutical preservative.

MATERIALS AND METHODS

Strains and Plasmids

Plasmid pMC614 contains the native yeast *MF*α1 signal sequence for the secretion of human lysozyme, while plasmid pMC632 employs the rat α-amylase signal sequence. These plasmids were transformed into the host, *Saccharomyces cerevisiae* strain SEY2101 (MAT α *ura3*-52 *leu2*-3, *leu2*-112, *his4*-519). The yeast transformants were screened for lysozyme production with the use of "lysoplates". These consisted of petri dishes with two different layers of agar. The first layer contained all the medium components required for growth of the transformants. The second layer covered the first and consisted of bacterial cells. The second agar layer was opaque and colonies of lysozyme-producing yeast cells formed a clear halo, while colonies that could not produce lysozyme grew but it did not form a halo. Most transformants that grew on these lysoplates produced human lysozyme.

Medium and Cultivation

The recombinant yeasts were grown in a defined minimal dropout-medium without leucine. The medium was composed of bacto yeast nitrogen base, excluding amino acids (6.7 g), glucose (70 g), 50× leucine-dropout stock solution (20 ml), and tyrosine (0.1 g) in a total 1.0 l volume. To prepare 50× leucine-dropout stock solution, tryptophan (0.25 g), arginine (0.20 g), histidine (0.60 g), isoleucine (0.60 g), lysine (0.60 g), methionine (0.10 g), phenylalanine (0.60 g), threonine (0.50 g), adenine (0.12 g), and uracil (0.24 g) were added in a 100 ml of water. Fermentations were carried out by incorporating a fully automated fermentation system (Mouse, Queue Systems, U.S.A.). A pH of 5.5 was maintained through a controlled addition of the filter-sterilized 1.0 M H_2SO_4 and 1.0 M NH_4OH. For aerobic cultures, dissolved oxygen (DO) at 90% of the saturation level was maintained by monitoring with a polarographic DO probe (Ingold, Switzerland) and making a partial change in an aeration rate with the help of an impeller speed. The optical density of cell cultures was measured at 600 nm with a spectrophotometer (Milton Roy, U.S.A.).

Analytical Methods

Glucose concentrations were determined with a glucose analyzer (Model 27, Yellow Springs Instruments, U.S.A.). Ethanol concentrations in the fermentation broth were determined with a gas chromatograph (Varian 3700, U.S.A.). For determining the amount of human lysozyme activity in either the culture broth or inside the cells, a method was developed by Morsky [14] in which an assay of human body fluids was adapted for use with the recombinant yeasts. In the assay, a sample with an unknown quantity of lysozyme was added to 55 mM phosphate buffer (pH 6.2), which contained human serum albumin, known as a stabilizer, and *Micrococcus lysodeikticus* (Sigma Chemicals, St. Louis, U.S.A.), identified as a substrate. The mixture was placed in a prewarmed cuvette (37°C) and the decrease in absorbance was followed for three min. Lysozyme activity was defined as the decrease in absorbance per minute at 700 nm (1 unit=0.001 DA700/min). To monitor the extracellular activity, 0.5 ml of broth samples were centrifuged at 16,000 ×g for 2 min and washed with 0.2 M ammonium acetate, because it has been reported that an appreciable amount of human lysozyme is ionically bound to the surface of the cells. At alternative times during the fermentation, 30 ml of samples were harvested by centrifugation, washed with 0.2 M ammonium acetate and stored at 4°C. The lysozyme activity inside the cells, periplasmic and cytoplasmic activity combined, was determined after lysis of the cells by glass beads.

Cell Fractionation

In order to determine what kinds of effect signal sequences have on the secretion of human lysozyme through the cytoplasm and the periplasm, the yeast cell wall was selectively degraded cautiously without rupturing the cell membrane. A modified spheroplasting method using Zymolyase 20T (ICN Biomedicals, Aurora, U.S.A.), as developed by Wolkda-Mitasko [18], has been described previously [9]. To verify whether the fraction method

worked correctly, the activity of a cytoplasmic marker protein, glucose-6-phosphate dehydrogenase (G-6-PDH), was examined in both the periplasmic and the cytoplasmic fractions. It was discovered that more than 90% of the G-6-PDH activity was contained in the cytoplasmic fraction, and this indicates that the fractions included the proteins as specified.

RESULTS AND DISCUSSION

Comparison of Signal Sequences

Many factors are proposed to exert some influences on either the efficiency or the kinetics of the secretion process in yeast. Our primary concerns are for the elements of the gene cassette including the promoter, signal sequence, structural gene and terminator, the location of the cassette on either the host cell's chromosome or a plasmid, and finally, the number of copies of the cassette in the cell and the genetic characteristics of the host strain. Among these, we studied to find out what kind of influence the signal sequence has on the secretion of human lysozyme. A batch fermentation was performed to measure how much lysozyme was secreted to the medium by the rat α-amylase signal sequence. The results obtained with the recombinant yeast harboring the plasmid PMC632 are shown in Fig. 1. The growth curve, glucose and ethanol concentrations, and human lysozyme profiles in the intracellular and extracellular fractions were displayed. Lysozyme secreted from the cells increased along with the cell growth owing to the constitutive promoter and then it slowed down. Eventually, it stopped as the cells stopped growing. The intracellular lysozyme level rose as the cell grew. The intracellular level of lysozyme begin to fall and reached a certain level only after the cells stopped growing, and the expression of lysozyme stopped. Specific growth rate during the exponential growth phase was 0.28 h^{-1} and the lysozyme secretion rate during this period was 5 units/ml/h.

Plasmid pMC614 carries the lysozyme structural gene (HLZ) expressed under the control of the *ADH1* promoter and the secretion is directed by the *MF*α1 native yeast signal sequence. The results with the recombinant yeast harboring plasmid pMC614 were depicted in Fig. 2. Overall fermentation behavior was very similar to that of the previous recombinant yeast except for the lysozyme secretion. Over the course of the fermentation period, the lysozyme activity contained inside the cells fell and eventually reached zero as the production of HLZ ceased and the pool of lysozyme in the secretion pathway exited the cell. The lysozyme outside the cell also exhibited a different behavior. Secretion of HLZ to the medium occurred almost immediately along with the cell growth. In addition, this could be contrasted with the non-native signal sequence

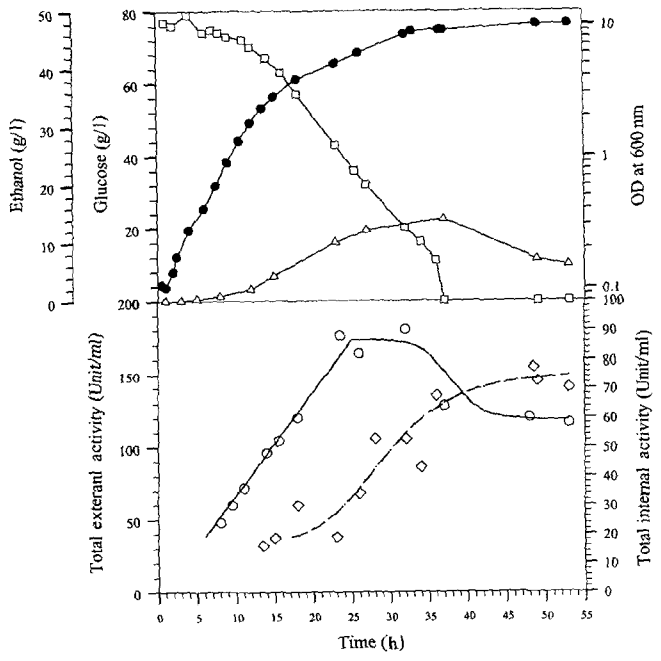

Fig. 1. Profiles of optical density (●), glucose concentration (□), ethanol concentration (△), and human lysozyme activities at extracellular (◇) and intracellular (○) compartments of yeast containing plasmid pMC632 (contains α-amylase signal sequence), grown in a batch fermentation at pH 5.5 and 30°C: The lines were drawn to show a trend.

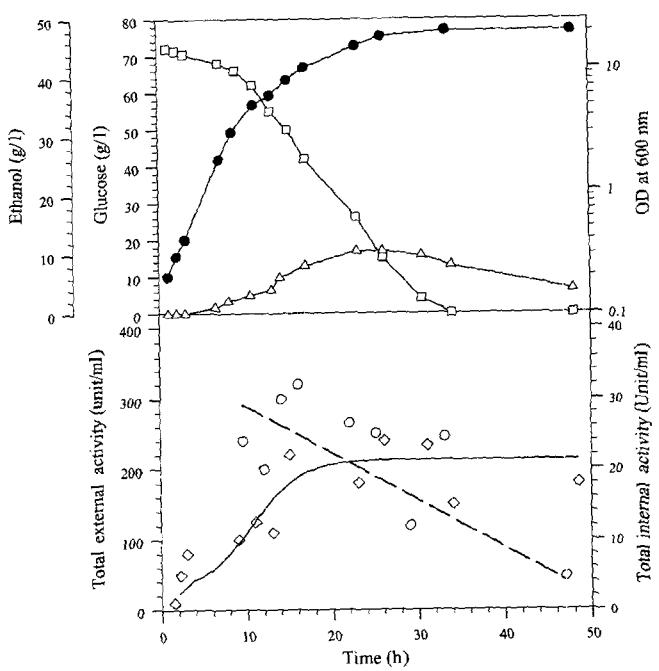

Fig. 2. Trajectories of optical density (●), glucose concentration (□), ethanol concentration (△), and human lysozyme activities at extracellular (◇) and intracellular (○) compartments of yeast containing plasmid pMC614 (contains *MF*α1 signal sequence), grown in a batch fermentation at pH 5.5 and 30°C.

Table 1. Growth and secretion of lysozyme for the recombinant yeast cells, each with the *MF*α1 signal sequence or the rat α-amylase signal sequence.

Plasmid	Signal sequence	Specific growth rate (h^{-1})	Lysozyme secretion rate (units/ml/h)	Maximum lysozyme activity (units/ml)
PMC614	*MF*α1	0.34	11.1	220
PMC632	α-amylase	0.28	4.9	150

by which secretion began after a rather long lag phase of about 10 h. The amount of product secreted out of the cell reached a maximum of about 200 units/ml, while that contained inside the cell showed a maximum lysozyme level of only 20 to 30 units/ml at the beginning of the experiment. This is in contrast to the lysozyme expressed by the non-native signal sequence, which showed a maximum lysozyme level of 100 units/ml at its highest point. It seems that the native yeast signal sequence was significantly more efficient for secreting the lysozyme compared to the non-native signal.

Table 1 shows that both the growth rate and the lysozyme secretion rate for the plasmid with the native yeast signal sequence were higher than for cells carrying the plasmid with the non-native signal sequence. The secreted lysozyme with a non-native signal sequence possibly got caught up in the secretion pathway, and this caused a block in this pathway leading to a build up of the translated protein, which in turn reduced the cell growth rate. It was also reported that the non-native signal sequence of *Kluyveromyces* inulinnase exhibited signs of being unstable when it was expressed with a human serum albumin in yeast [7]. Based on the result mentioned above, it seems to be a reasonable way to choose a native signal sequence, such as *MF*α1, *SUC2*, and so on, for secretion of heterologous proteins in yeast.

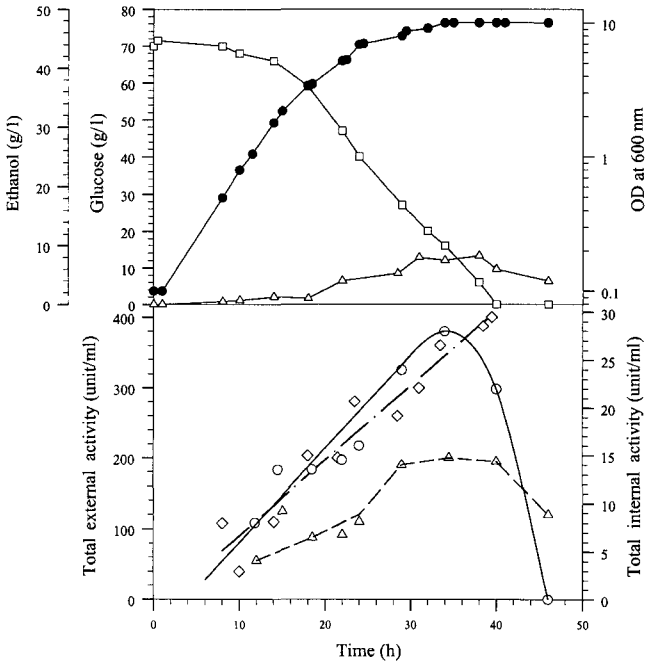

Fig. 3. Fractionation of human lysozyme activity in the recombinant yeast.
pMC614 with the *MF*α1 signal sequence grown at pH 5.5 and 30°C; optical density (●), glucose concentration (□), ethanol concentration (△), extracellular (◇), periplasmic (△), and cytoplasmic (○) human lysozyme activities.

Comparison of Protein Sizes

In order to determine the sizes of proteins secreted through the cytoplasm and the periplasm, the yeast cell carrying the human lysozyme gene fused to the *MF*α1 signal sequence was grown in the medium, and the content of lysozyme at each fraction was measured after fractionation of cells (Fig. 3). The recombinant yeast showed an exponential growth for the first 20 h of the fermentation. Even at a high level of glucose concentration at 60 g/l, cell growth began to slow down. Towards the end of the fermentation, the ethanol concentration approached to 10 g/l, which is still below the reported concentration that inhibits cell growth. The lysozyme activity profiles showed that the enzyme concentrations in all three spaces, extracellular, periplasmic and cytoplasmic, increased as the cells grew. Levels of lysozyme in the cytoplasmic and periplasmic spaces dropped dramatically when the cells stopped growing, and thus stopped producing the enzyme. In effect, an internal store of the lysozyme was depleted as the lysozyme expression ceased and the pool was secreted outside the cell. There was a big difference in the amount of lysozyme retained in either the periplasm or cytoplasm versus outside the cell. This indicated that the secretion of this protein was extremely efficient and that only a small percentage of the product was retained in the periplasmic space. Meanwhile, our previous study with invertase [10] conducted under the same culture condition showed an opposite trend in secretion. The yeast invertase had a mass of 270 kDa in its glycosylated form and it was an order of magnitude larger than lysozyme. The secretion rate of invertase by the *MF*α1 signal sequence was not efficient, leaving over 60% of total invertase activity in the periplasmic space. This comparison suggests that protein size may affect the secretion efficiency. Similar results were also previously reported in an experiment using a different protein [20]. However, the degree of effect caused by glycosylation on the protein was not clearly distinguished by this experiment.

Effect of Dissolved Oxygen

Environmental factors including temperature, pH, and dissolved oxygen may influence the secretion process. Since oxygen content in the medium greatly affect yeasts physiology and metabolic rates, the effect of oxygen on the secretion behavior of yeast was closely examined. A batch fermentation was performed under an anaerobic condition to determine whether this parameter had any effect on the cell growth and lysozyme secretion in cells carrying plasmid pMC614. The growth parameters and lysozyme activity profiles are shown in Fig. 4. Ethanol production reached a level of about 30 g/l as compared to about 10 g/l for the aerobic growth in Fig. 2. The cell growth rate of an anaerobic culture (0.35 h^{-1}) was not significantly altered compared with an aerobic culture (0.34 h^{-1}). As the cells grew, the amount of lysozyme increased and then stopped rising as the cells stopped growing. The lysozyme activity inside the cells decreased during the course of fermentation until it reached to zero when the cells stopped growing. There was a large difference in the total amount of lysozyme produced: In the aerobic experiments, the total extracellular lysozyme reached a level of about 200 units/ml, while in the anaerobic experiment, the level reached almost twice with a value of about 400 units/ml. This difference was accounted for by the promoter which expressed lysozyme in the cells.. The *ADH*I promoter usually expresses a gene product that is on the fermentative pathway in yeast and is expressed more strongly as more carbon flux is forced down in this pathway. Thus, as the cells grew anaerobically, and more carbon flux was directed towards the fermentative pathway, the promoter caused more lysozyme to be produced and secreted, while the growth rate was not adversely affected. However, the lysozyme secretion rate was still 11 units/ml/h, which was almost identical to that of the cells when grown aerobically. This result indicates that the secretion rate of the protein is not greatly varied by the dissolved oxygen content in the media and consequently by the expression rate inside the cells.

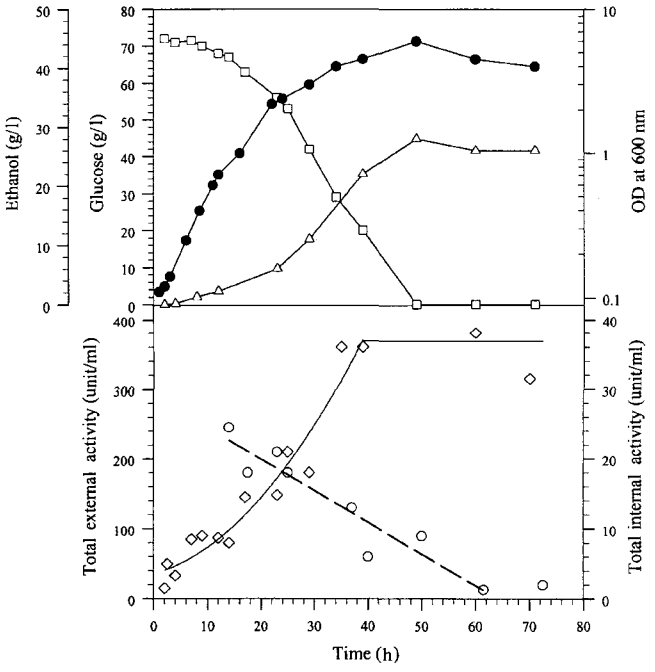

Fig. 4. Fermentation profiles of the recombinant yeast.
pMC614 grown in an anaerobic culture at pH 5.5 and 30°C; optical density (●), glucose concentration (□), ethanol concentration (△), extracellular (◇), and intracellular (○) human lysozyme activities.

CONCLUSIONS

To investigate the secretion behavior of human lysozyme (HLZ) from *Saccharomyces cerevisiae*, a native *MF*α1 signal sequence and a non-native rat α-amylase signal sequence were fused and expressed by the *ADH*I promoter. The native yeast signal sequence was more efficient in the secretion of lysozyme than in the non-native signal saguence. Both the growth rate and the lysozyme secretion rate for the plasmid with a native yeast signal sequence were higher than the non-native signal sequence. By comparing the secretion behavior of both lysozyme and yeast invertase under the direction of the *MF*α1 yeast signal sequence, it was suggested that the peptide size might affect the secretion efficiency in yeast. Lysozyme was efficiently secreted outside the cell, while invertase with a larger molecular size was retaining over 60% of the total activity in the periplasmic space. When expressed at an anaerobic condition where the *ADH*I promoter is derepressed, the amount of lysozyme secretion was more than that of the aerobic culture, but the secretion rates were identical regardless of the dissolved oxygen level in the medium. This observation suggested that the secretion efficiency is dependent upon a dissolved oxygen content which, in turn, changed the expression rate of lysozyme by using the *ADH*I promoter.

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