Note

Comparison of Three Signals for Secretory Expression of Recombinant Human Midkine in Pichia pastoris

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Received March 7, 2001; Accepted June 6, 2001

The secretion signals of Saccharomyces cerevisiae α mating factor, human midkine itself, and Pichia pastoris acid phosphatase, were tried for the expression of human midkine under the control of the AOX1 gene promoter in P. pastoris. Approximately 28 mg/l, 1.5 mg/l, and 0.2 mg/l of midkine were secreted by using the α mating factor pre-pro-sequence, the midkine signal sequence, and the phosphatase signal sequence in flask cultures, respectively.

Key words: pre-pro-sequence; signal peptide; secretory expression; human midkine; Pichia pastoris

Midkine is one of the heparin-binding proteins, the synthesis of which is regulated in the course of development. On the other hand, it regulates the growth, survival, and differentiation of cells.1) Recently, the methylotrophic yeast, Pichia pastoris, has been widely used in many works of protein expression in cells or secreted into the medium.2) Human serum albumin is successfully expressed using its pre-pro-sequence.3) Midkine is expressed into the medium using the midkine signal peptide6) in P. pastoris. It was accurately processed, and showed its biological activities. However, the level of expression was not very high. One possible approach may be to find an appropriate secretion signal for a high amount of expression. We tried to use the pre-pro-sequence of α mating factor5) from yeast, and the signal sequence of an acid phosphatase4) from P. pastoris in addition to the signal sequence of human midkine for the expression of human midkine in P. pastoris.

The midkine cDNAs were prepared for the three kinds of expression vectors described below, by a PCR method using the cloned midkine cDNA6) as a template. For the secretion of human midkine using its own signal sequence, the expression vector of pHILD4 (Invitrogen, San Diego, CA, USA) was used.7) The cDNA coding for the midkine precursor including the signal peptide,4) which had the recognition sequence for EcoRI in both ends, was digested with EcoRI, and inserted into the EcoRI site immediately downstream of the AOX1 promoter as shown in Fig. 1(B). For the secretion using α factor pre-pro-sequence, the expression vector, pPIC9K (Invitrogen), was used, which contained the sequence coding for the pre-pro-sequence immediately downstream of the AOX1 promoter. The cDNA coding for mature midkine6) was inserted, as described above, into the EcoRI site at the 3′ end of the sequence coding for the pre-pro-sequence (Fig. 1(A)). For secretion using the phosphatase signal sequence, the expression vector, pHILS1 (Invitrogen), was used, which contained the sequence coding for the signal peptide immediately downstream of the AOX1 promoter. The cDNA coding for the mature midkine was inserted, as described above, into the EcoRI site at the 3′ end of the sequence coding for the signal peptide (Fig. 1(C)).

The host, P. pastoris GS115 (his4) (Invitrogen), was transformed4,8) with each of three kinds of expression plasmids, separately. The HIS transformants were screened.8) The expression vectors, pPIC9K and pHILD4, contain kanamycin resistance genes from E. coli immediately upstream of HIS4 genes. The transformants with pPIC9K, and pHILD4 expression plasmids were confirmed to have an expression cassette on the plates containing 0.5 mg/ml G418. Fifteen clones were randomly selected from the transformants integrated with the pPIC9K expression plasmid, 56 clones from the transformants integrated with the pHILD4 expression plasmid, and 50 clones from the transformants integrated with the pHILS1 expression plasmid. For the comparison of the midkine expression in these transformants, they were grown overnight at 30°C in fermentation medium containing 4% (v/v) glycerol using shaking flasks, and then, transferred to the medium containing 1% (v/v) methanol in order to induce midkine expression at 30°C for three days.9) The cells were removed from the culture broth by centrifugation, and the concentration of midkine in the medium was measured by ELISA, using chemically...
A factor pre-pro-sequence

\[ \text{XhoI} \quad \text{SnaRI} \quad \text{EcoRI} \rightarrow \text{Mature midkine} \]

-CTC GAG AAA AGA GAG GCT GAA GCT TAC GTA GAA TTC AAA AAG AAA GAT--
-Leu Glu Lys Arg Glu Ala Glu Ala Tyr Val Glu Phe Lys Lys Asp--

\[ \text{KEX2 site} \quad \text{Spacer/Cleavage sites} \]

AOXI promoter

\[ \text{EcoRI} \rightarrow \text{Midkine cDNA} \]

---CACTAATTATCGAAGCCAGGAATTC ATG CAG CAC CGA GGC TTC CTC CTC---

Met Gln His Arg Gly Leu Leu Leu---

PHO1 signal sequence

\[ \text{EcoRI} \rightarrow \text{Mature midkine} \]

--TTG GCT ACT TTG CAA TCT GTC TTC GCT CTA GAA TTC AAA AAG AAA---
-Leu Ala Thr Leu Gln Ser Val Phe Ala Arg Glu Phe Lys Lys---

\[ \text{Signal peptide cleavage site} \]

Fig. 1. Nucleotide Sequence and Amino Acid Sequence Around the Junction of Expression Vector and Coding DNA for Midkine.

The junction of the coding sequence for human midkine and pPIC9K (A), pHILD4 (B), or pHLIS1 (C) is shown. The solid triangle indicates the cleavage site, and the arrow shows the start point of the cDNA for human midkine. The recognition sequences for restriction enzymes are indicated by the lines over the nucleotide sequences. The spacer of \( \alpha \) factor signal, Glu-Ala-Glu-Ala, is underlined.

The synthesized human midkine (Peptide Institute, Inc., Osaka, Japan) was used as the standard.

Midkine expressions in the five best transformants from each group are shown in Fig. 2. Most of these transformants may have a single copy of expression cassette in their chromosomes, because these were not selected for a high copy number of the cassette. The higher copy number transformants using \( \alpha \) mating factor pre-pro-sequence, or midkine signal sequence, which are selected on the plates containing 1 mg/ml or 2 mg/ml G418,\(^{[10]}\) are not able to express higher amounts of midkine. Therefore, the amount of midkine expression may depend on the secretion signals. The best transformant, using \( \alpha \) mating factor pre-pro-sequence, expressed 28 mg/l of human midkine in a shake flask culture (Fig. 2). The primary transformants obtained with \( \text{HIS} \) screening may include Mut\(^{+}\), Mut\(^{-}\), or Mut\(^{-}\) strains. However, the transformants selected with the expression experiments described above, and not selected for Mut\(^{+}\) or Mut\(^{-}\), will be Mut\(^{+}\). The best transformant obtained here was a Mut\(^{+}\) strain, in which the \( \text{AOXI} \) gene had not been disrupted with the expression cassette by the gene replacement, and the \( \text{AOXI} \) gene was actively expressed.

The \( \alpha \) mating factor secretion signal used for the best transformant has the pre- and pro-sequences. Another two signals from midkine and phosphatase are the signal peptides, and do not have the pro-sequence. Signal peptide or pre-sequence have important roles in the interaction with the signal recognition particle, and in the translocation of the nascent polypeptide to the endoplasmic reticulum. However, in some cases the pro-sequence governs the efficiency of the protein secretion.\(^{[11,12]}\) The results of the secretion described above show that the long pro-sequence of the \( \alpha \) mating factor signal may be more important in the secretory expression of human midkine. The pro-sequence may promote the translocation of midkine polypeptide to the Golgi complex, or to the secretion vesicle. Another possibility is that the pro-sequence may be especially important to assist the folding or the formation of disulfide bonds in a molecule,\(^{[13]}\) because the authentic midkine have a very compact three-dimensional structure formed.
with the precisely linked five disulfide bonds in each molecule.\(^{14}\)

The secreted protein needs neither to be dissolved with urea, or a guanidine hydrochloride solution, nor to be refolded \textit{in vitro}. The ecological and also the economical production of midkine will be possible using the \(\alpha\) mating factor pre-pro-sequence in \textit{P. pastoris}.

\section*{Acknowledgment}

We wish to thank Prof. T. Muramatsu, and Dr. K. Kadomatsu in Nagoya University for providing us human midkine cDNA.

\section*{References}


