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An Approach to the Removal of Yeast Specific *O*-Linked Oligo-Mannoses from Human Midkine Expressed in *Pichia pastoris* Using Site-Specific Mutagenesis

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Human midkine is expressed and secreted in the medium under the control of an *AOX1* gene promoter in *Pichia pastoris* using its own secretion signal. The midkine precursor is properly processed to yield the correct amino-terminus of mature midkine. However, more than half of the product receives yeast specific mannosylations. The sites for the mannosylations were determined to be the three threonine residues in the carboxy-terminal region of human midkine. In order to obtain non-mannosylated midkine, alanine residues were substituted for the three threonine residues by site specific mutagenesis. HPLC and mass spectrometry confirmed that the mutant midkine contained almost no mannose residues. Despite the amino acid substitutions in the carboxy-terminal region, mutant human midkine, promoted CHO cell proliferation as well as normal midkine.

Key words: human midkine, *O*-mannosylation, *Pichia pastoris*, secretion, site-specific mutagenesis.

Midkine is one of the heparin-binding proteins, the syntheses of which are regulated during the course of the development. On the other hand, midkine itself regulates the growth, survival, and differentiation of cells. Human midkine consists of 121 amino acids with a molecular mass of 13,240 Da (*1*, *2*). Human midkine is extremely basic because it contains 23 lysine and 7 aginine residues. The whole human midkine molecule has been chemically synthesized and renatured (*3*). Midkine is composed of two domains with intra-domain disulfide bridges (*4*). The amino-terminal and carboxy-terminal domains have been chemically synthesized independently, and analyzed by NMR in aqueous solution (*5*). This revealed that both domains consist of three anti-parallel β-strands. Human midkine does not have the consensus amino acid sequence for *N*-linked glycosylation, and it has not been reported that the native form of midkine contains *O*-linked glycosyl residues. Therefore, it is believed that "authentic" midkine is not glycosylated at all.

A heterologous protein expression system in *Pichia pastoris* has been used to produce many proteins in cells, or in the medium by high cell-density fermentation (*6*, *7*). For example, human serum albumin (4 g/liter) (*8*), and insulin-like growth factor I (1 g/liter) (*9*) have been successfully secreted into the medium. In this study, we tried to produce midkine using the *P. pastoris* expression system, and the sugar modifications of the product were studied.

MATERIALS AND METHODS

Materials—*Eco*RI restriction enzyme, and 4-vinyl-pyridine were purchased from Takara (Kyoto). *P. pastoris*, GS115 (*his4*), and the expression vector pHILD4 were provided by Phillips Petroleum (Bartlesville, OK, USA). pcDNA3 was purchased from Invitrogen (San Diego, CA, USA). Bovine serum albumin and G418 were purchased from Sigma (Tokyo), and α-D-methyl mannopyranoside was from Wako (Osaka). Chemically synthesized human midkine was purchased from Peptide Institute (Osaka), and immobilized V8 protease was from Pierce (Rockford, IL, USA). SP-Sepharose, HiTrap Heparin, and ConA-Sepharose CL4B were purchased from Amersham-Pharmacia Biotech (Tokyo). DMEM medium was obtained from Nissui Pharmaceutical (Tokyo), Nu-serum type IV was from Collaborative Biomedical Products (Bedford, MA, USA), and the transfection kit was from Stratagene (La Jolla, CA, USA).

Construction of Expression Plasmids—The expression plasmid consisted of an expression vector and midkine cDNA. The cDNA inserted into an expression vector was prepared by a PCR method. Recognition sequences for *Eco*RI were added to both outer ends of the cDNA coding sequence for the midkine precursor. Cloned human midkine cDNA (*10*) was used as a template for PCR. The upstream primer was 5′-GCGGAATTCATGCAGCACCGAGGCTTCCTC-3′. The downstream primer was 5′-GCGGAATTCCTAGTCCTTTCCCTTCCCTTT-3′. To prepare the expression plasmid for *P. pastoris*, the amplified midkine cDNA was digested with *Eco*RI, and inserted into the *Eco*RI site of pHILD4, located immediately downstream of the *AOX1* promoter (*11*).

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For the expression of human midkine in COS7 cells, the expression vector pcDNA3 was used. The expression plasmid was constructed in a similar way to the construction of the *P. pastoris* expression plasmids. The cDNA coding for the midkine precursor was inserted into the *Eco*RI site of the vector, located immediately downstream of the cytomegalovirus early promoter and enhancer region.

Transformation of Host Cells and Expression of Midkine—Transformation of *P. pastoris*, GS115 (*his4*) by electroporation, and the selection of the transformants were performed according to the instructions of the suppliers. Transformants were grown overnight in test tubes in basal fermentation medium (*12*) supplemented with 4% glycerol at 30°C with shaking, and the cells were then transferred to basal medium containing 1% methanol, and cultured for three days at 30°C with shaking to induce the expression of midkine. Once per day during induction methanol was added to each tube (finally 1%), and the pH was adjusted to 5. The same method was applied to shake-flask culture containing one-liter of medium, except that the cells were induced for production at 20°C for 36 h.

Human midkine was transiently expressed in COS7 cells. Cells were transfected by the DEAE dextran method with an expression plasmid consisting of pcDNA3 and midkine cDNA. After transfection, the cells were incubated for the expression of midkine in DMEM-10% Nu-serum at 37°C under an atmosphere of 5% carbon dioxide for three days.

High Cell-Density Fermentation of P. pastoris—High cell-density fermentations were performed according to the procedure described by Clare, J.J. *et al.* (*12*) with minor modifications. The culture volume at the start of fermentation was 3.5 liters, and the cells were induced to express midkine at 20°C and pH 5.8 with the continuous addition of methanol. The methanol feeding rate was gradually increased from about 1.4 g/h to about 16 g/h over a period of 3 days.

Purification of Human Midkine—An SP-Sepharose column and HiTrap heparin column were used for the purification of recombinant midkine. The columns were equilibrated with 0.15 M NaCl, 50 mM sodium phosphate, pH 6.0. The sample was applied to the column, and the column was washed with 0.5 M NaCl, 50 mM sodium phosphate, pH 6.0. Midkine was eluted with 2 M NaCl, 50 mM sodium phosphate, pH 6.0.

Determination of Midkine Concentration—A sandwich ELISA system (*13*) was constructed using a rabbit anti–human midkine antibody and the chicken anti–human midkine antibody conjugated with horseradish peroxidase.

Determination of Sugars in Midkine—Oligosaccharides present in preparations of purified midkine expressed in *P. pastoris* were hydrolyzed into monosaccharides and subjected to fluorescence labeling (*14*). Sugars were then quantitated by HPLC.

Isolation of a Mannosylated Peptide by Complete Digestion of Midkine with V8 Protease—Purified midkine expressed in *P. pastoris* (3.6 mg) was denatured, modified with 4-vinyl-pyridine, and then completely digested with immobilized V8 protease according to the instructions of the suppliers. The digestion products in 0.5 M NaCl, 50 mM Tris-Cl, pH 7.5, were applied to a ConA-Sepharose column. Peptide bound specifically to the column was eluted with α-D-methylmannoside and subjected to SDS–polyacrylamide gel electrophoresis (*15*), transferred to a PVDF membrane (*16*), and stained with Coomassie Brilliant Blue.

Mutagenesis of Human Midkine—The cDNA coding for mutant midkine was synthesized by PCR. Cloned normal midkine cDNA was used as a template. The upstream primer was 5′-CCGAATTCATGCAGCACCGAGGCTTCCTCC-3′. The long downstream primer was also chemically synthesized, and had the sequence 5′-CCGAATTCTTAGTCCTTTCCCTTCCCTTTCTTGGCTTTGGCCTTTGCTTTAGCCTTGGGGGTGCAGGGCTTAGCGACGCGGATAGCCTCCTGGC-3′. The nucleotide triplet, 9–11, is a translation terminator. Nucleotides 51–53, 72–74, and 84–86 code for alanines instead of threonines in the normal midkine cDNA. Different nucleotides in the downstream primer compared with normal midkine cDNA are underlined.

Other Methods—The amino acid sequence was determined using a protein sequencer, PSQ-1 (Shimadzu, Kyoto) or G1005A (Hewlett-Packard, Palo Alto, CA, USA). Mass spectrometry (MALDI-TOF) was performed with Vision 2000 (Finnigan, Thermo Quest, Austin, TX, USA) or Voyager Elite (PerCeptive Biosystems, Foster City, CA, USA) calibrated with the external standards ubiquitin and myoglobin. For HPLC analysis, a Shimadzu LC10 liquid chromatography system was used.

RESULTS AND DISCUSSION

Comparison of Human Midkine Expressed in P. pastoris with That Expressed in Mammalian Cells—Midkine expressed in COS7 cells showed a single peak on HPLC analysis (Fig. 1A) using a reverse phase column. Human midkine was also produced by *P. pastoris*. In this case, the secretion signal of midkine itself seemed to work very well in *P. pastoris*. In fact, up to 0.1 g/liter-medium of midkine was secreted in high cell density fermentation. Midkine was purified by the standard procedure. The purified midkine showed at least 6 peaks on HPLC analysis (Fig. 1B), and showed smeared double bands when the proteins were stained after SDS–polyacrylamide gel electrophoresis (data not shown). These double bands were also stained in Western blot analysis. The N-terminal sequence of purified midkine was determined to be Lys-Lys-Lys-Asp-Lys-Val-Lys-Lys-Gly-Gly, the same as that of mature midkine (*11*). Peaks seen in the HPLC analysis of purified midkine may be due to midkine itself and derivatives of midkine, rather than contaminating proteins. The proteins in peaks M5, M1, and M0 in Fig. 1B were isolated and subjected to mass spectrometry. The average masses were 14,051, 13,401, and 13,238 Da for the proteins in peaks M5, M1, and M0, respectively. The theoretical molecular mass of midkine is 13,240.3 Da. The protein in peak M0, and midkine transiently expressed in COS cells (Fig. 1A), seems to be non-modified authentic midkine, and the proteins in peaks M5 and M1 may be midkine molecules with five and one monosaccharide residues, respectively, based on the molecular masses.

One mole of midkine contains 3.7 mol of mannose, 0.3 mol of glucose, and 0.1 mol of xylose on average. The amount of glucose covalently bound to midkine might be less than 0.3 mol/mol, because the glucose residues might be contaminated during purification. About half of the midkine bound to ConA Sepharose column under the conditions used for the isolation of a mannosylated peptide

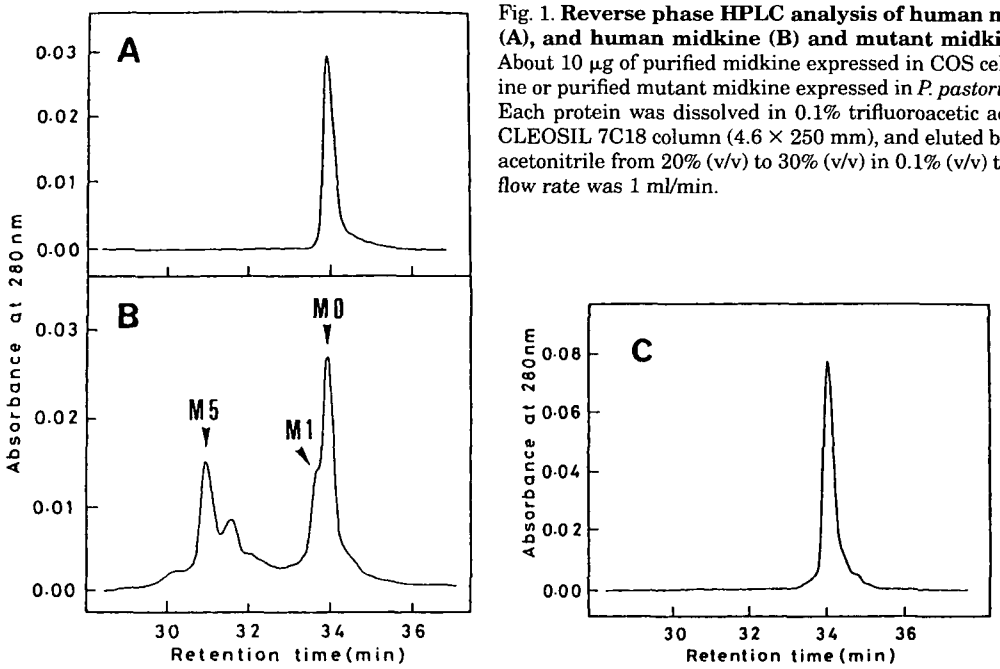

Fig. 1. **Reverse phase HPLC analysis of human midkine expressed in COS7 cells (A), and human midkine (B) and mutant midkine (C) expressed in *P. pastoris*.** About 10 μg of purified midkine expressed in COS cells, or about 30 μg of purified midkine or purified mutant midkine expressed in *P. pastoris* was submitted to HPLC analysis. Each protein was dissolved in 0.1% trifluoroacetic acid (v/v), applied to a Chemco NUCLEOSIL 7C18 column (4.6 × 250 mm), and eluted by a linear concentration gradient of acetonitrile from 20% (v/v) to 30% (v/v) in 0.1% (v/v) trifluoroacetic acid (15–40 min). The flow rate was 1 ml/min.

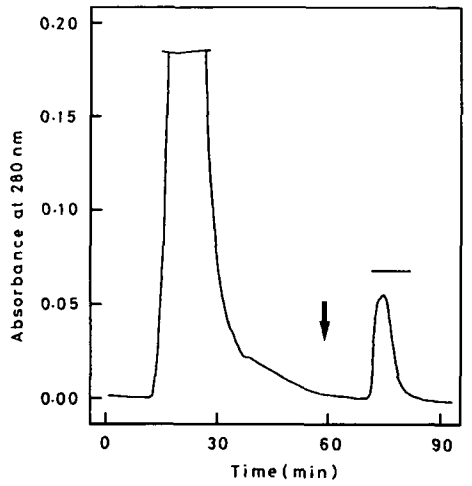

Fig. 2. **Isolation of a mannosylated peptide by Con A–Sepharose column chromatography from the peptides resulting from the complete digestion of midkine with V8 protease.** Two thirds of the digested protein, 2.4 mg, was applied. The arrow indicates the start of the elution with 0.5 M α-D-methyl mannopyranoside. The bar over the peak around 75 min indicates the collected fractions. The flow rate was 0.4 ml/min.

1 20 40
KKKDKVKKGGPGSE▼CAE▼WAWGPCTPSSKDCGVGFRE▼GTCG
60 80
AQTQRIRCRVPCNWKKE▼FGADCKYKFE▼NWGACDGGTGTKV
100 121
RQGTLKKARYNAQCQE▼**T**IRV**T**KPCTPK**T**KAKAKAKKGKGKD

Fig. 3. **Peptide bonds cleaved by V8 protease, and mannosylated amino acids in human midkine expressed in *P. pastoris*.** The solid triangles show the peptide bonds cleaved by V8 protease, and the bold capitals in the carboxy-terminal fragment show the mannosylated amino acids.

described below. When heterologous proteins are expressed in *P. pastoris*, it is known that they receive yeast specific *O*-mannosylations in some cases (*7*, *9*). These phenomena have also been observed in heterologous proteins expressed in *Saccharomyces cereviciae* (*17–19*). It is plausible that the above described human midkine expressed in *P. pastoris* also received yeast specific *O*-mannosylations, although the detailed structures of the sugar linkages, and the mechanism of the sugar addition have not been identified for *P. pastoris* *O*-mannosylations (*7*).

Mannosylation Sites in Midkine—A mannosylated peptide resulting from the complete digestion of midkine with V8 protease was isolated (Fig. 2). Only one kind of peptide was obtained: a peptide with an amino acid sequence determined to be X-Ile-Arg-Val-X-Lys-Pro-X-Thr-Pro-Lys-X, indicating that the peptide was derived from the carboxy-terminus of human midkine (Fig. 3).

The amino acids in the X-positions may be modified, because almost all the X-position amino acids resulting from Edman degradation of the peptide were nonstandard amino acids. The X's, other than the third, are consistent with the positions of threonine residues in the carboxy-terminal peptide. The third X may be a modified cysteine residue, because its position is consistent with that of the cysteine residue in the carboxy-terminal peptide, and because all the cysteine residues in the purified midkine had been modified with 4-vinyl-pyridine. The three threonine residues described above seemed to contain mannose modifications. As shown in Fig. 3, mannosylated threonine residues were located next to positively or negatively charged amino acid residues. *O*-Glycosylation sites in the recombinant B-chain of platelet-derived growth factor expressed in *Saccharomyces cerevisiae* also have positively or negatively charged amino acids nearby (*18*). This may be required for the *O*-glycosylation of threonine or serine in yeast for the access of *O*-glycosyl transferases. The important role of charged amino acids in threonine *O*-mannosylation in mid-

kine is now being investigated.

Mutagenesis of Midkine cDNA and the Expression of Non-Mannosylated Midkine—If a protein is to be used as a medicine, it must be purified completely without any contaminants. When a human protein that is a simple and non-glycosylated protein in its natural state, is produced using a yeast protein expression system, it may be mannosylated. If so, the non-mannosylated protein has to be isolated from protein with even small amounts of mannose. However, this is difficult to accomplish efficiently. In this case, it is very important to express the protein without mannosylation.

Three threonine residues, thr 97, 101, and 108, in mature midkine (Fig. 3) seem to be mannosylated. Therefore, these threonine residues were replased with alanine residues by site-specific mutagenesis, and the mutated midkine was produced by *P. pastoris*.

One of the clones expressed about 3 μg/ml of mutant midkine in test tube culture. From one-liter medium in shake flask culture of this clone, about 1.6 mg of mutant midkine was purified by the standard procedure. The purified mutant midkine was analyzed by reverse phase HPLC as shown in Fig. 1C. Mutant midkine eluted as a single peak with the same retention time observed for fraction M0 (Fig. 1B). The molecular mass of mutant midkine was determined to be 13,149 Da by mass spectrometry, which is almost the same as the theoretical mass of mutant midkine (13,150 Da). No other signal for a higher molecular weight species was detected, suggesting that almost none of the mutant midkine molecules were modified by mannoses or other sugar molecules. It is also confirmed that the mannosylated amino acids in midkine expressed in *P. pastoris* are only the three threonine residues in the carboxy-terminal region.

One of the activities of midkine is to promote cell proliferation as described by Ratovitski *et al.* (*20*). Although the three threonine residues in the carboxy-terminal region of midkine were replaced by alanine residues, mutant midkine was able to promote CHO cell proliferation as well as normal midkine (data not shown).

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REFERENCES

1. Muramatsu, T. (1994) The midkine family of growth/differentiation factors. *Dev. Growth Differ.* **36**, 1–8
2. Kurtz, A., Schulte, A.M., and Wellstein, A. (1995) Pleiotrophin and midkine in normal development and tumor biology. *Crit. Rev. Oncogenesis* **6**, 151–177
3. Inui, T., Bodi, J., Kubo, S., Nishio, H., Kimura, T., Kojima, S., Maruta, H., Muramatsu, T., and Sakakibara, S. (1996) Solution synthesis of human midkine, a novel heparin-binding neurotrophic factor consisting of 121 amino acid residues with five disulphide bonds. *J. Pept. Sci.* **2**, 28–39
4. Fabri, L., and Maruta, H., Muramatsu, H., Muramatsu, T., Simpson, R.J., Burgess, A.W., and Nice, E.C. (1993) Structural characterization of native and recombinant forms of the neurotrophic cytokine MK. *J. Chromatogr.* **646**, 213–225
5. Iwasaki, W., Nagata, K., Hatanaka, H., Inui, T., Kimura, T., Muramatsu, T., Yoshida, K., Tasumi, M., and Inagaki, F. (1997) Solution structure of midkine, a new haparin-binding growth factor. *EMBO J.* **16**, 6936-6946
6. Higgins, D.R. and Cregg, J.M. (1998) Introduction to *Pichia pastoris* in *Methods in Molecular Biology* (Higgins, D.R. and Cregg, J.M., eds.) Vol. 103, pp. 1–15, Humana Press, Totowa, NJ
7. Cregg, J.M. (1999) Expression in the methylotrophic yeast *Pichia pastoris* in *Gene Expression Systems* (Fernandez, J.M. and Hoeffler, J.P., eds.) pp. 157–191, Academic Press, San Diego, CA
8. Bar, K.A., Hopkins, S.A., and Sreekrishna, K. (1992) Protocol for efficient secretion of HSA developed from *Pichia pastoris*. *Pham. Eng.* **12**, 48–51
9. Brierley, R.A. (1998) Secretion of recombinant human insulin-like growth factor I (IGF-I) in *Methods in Molecular Biology* (Higgins, D.R. and Cregg, J.M., eds.) Vol. 103, pp. 149–177, Humana Press, Totowa, NJ
10. Tsutsui, J., Uehara, K., Kadomatsu, K., Matsubara, S., and Muramatsu, T. (1991) A new family of heparin-binding factors: Strong conservation of midkine (MK) sequences between the human and the mouse. *Biochem. Biophys. Res. Commun.* **176**, 792–797
11. Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J-T, Smith, P.L., Wierschke, J.D., Subramaniam, A., and Birkenberger, L.A. (1997) Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast *Pichia pastoris*. *Gene* **190**, 55–62
12. Clare, J.J., Rayment, F.B., Ballantine, S.P., Sreekrishna, K., and Romanos, M.A. (1991) High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Bio/Technology* **9**, 455–460
13. Dusemund, B. and Barrach, H.J. (1982) Double-antibody enzyme-linked immunosorbent microassay for quantification of collagen types I and II. *J. Immunol. Methods* **50**, 255–268
14. Ohara, K., Sano, M., Kondo, A., and Kato, I. (1991) Two-dimensional mapping by high-performance liquid chromatography of pyridylamino oligosaccharides from various glycosphingolipids. *J. Chromatogr.* **586**, 35–41
15. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
16. Towbin, H., Staehelin, T., and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350–4354
17. Hard, K., Bitter, W., Kamerling, J.P., and Vliegenthart, J.F.G. (1989) O-Mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in *Saccharomyces cerevisiae*. *FEBS Lett.* **248**, 111–114
18. Settineri, C.A., Medzihradszky, K.F., Masiarz, F.R., Burlingame, A.L., Chu, C., and George-Nascimento, C. (1990) Characterization of O-glycosylation sites in recombinant B-chain of platelet-derived growth factor expressed in yeast using liquid secondary ion mass spectrometry, tandem mass spectrometry and Edman sequence analysis. *Biomed. Environ. Mass Spectrom.* **19**, 665–676
19. Ernst, J.F., Mermod, J.-J., DeLamarter, J.F., Mattaliano, R.J., and Moonen, P. (1987) O-Glycosylation and novel processing events during secretion of α-factor/GM-CSF fusions by *Saccharomyces cerevisiae*. *Bio/Technology* **5**, 831–834
20. Ratovitski, E.A., Kotzbauer, P.T., Milbrandt, J., Lowenstein, C.J., and Burrow, C.R. (1998) Midkine induces tumor cell proliferation and binds to a high affinity signaling receptor associated with JAK tyrosin kinases. *J. Biol. Chem.* **273**, 3654–3660