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Secreted expression of pseudozymogen forms of recombinant matriptase in *Pichia pastoris*

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ABSTRACT

Matriptase is a transmembrane serine protease expressed in vertebrates. This enzyme is synthesized as a zymogen form and is converted to an active form by cleavage at the N-terminus of the serine protease catalytic domain. In a mammalian cell-based expression system, we have produced pseudozymogen forms of recombinant matriptase (r-matriptase) that are activated by cleavage with a recombinant enterokinase (r-EK) *in vitro*. In the present study, four different pseudozymogen forms of r-matriptase containing a site for activation by r-EK and a hexahistidine tag (His_6-tag) were expressed in and secreted by *Pichia pastoris*, a methylotrophic yeast. The pseudozymogens with His_6-tag at their C-termini formed multimers linked by intermolecular disulfide bonds. After treatment with r-EK, they exhibited no detectable hydrolytic activity toward a chromogenic substrate. A pseudozymogen form of matriptase catalytic domain with N-terminal His_6-tag (designated His_6t-S-CD) was secreted as a monomer. His_6t-S-CD after r-EK treatment exhibited activity comparable to that of the activated form of an r-matriptase expressed in mammalian cells. His_6t-S-CD could be purified from culture medium in milligram quantities. The expression in the yeast offers an efficient method of producing larger amounts of r-matriptase.

1. Introduction

Matriptase (also known as membrane-type serine protease 1, epithin, *etc.*) is a membrane-bound serine protease found in vertebrates [1–4]. This enzyme belongs to type II transmembrane serine proteases, which are characterized by the N-terminal cytoplasmic domain, a signal anchor transmembrane domain, and an extracellular C-terminal serine protease catalytic domain (Fig. 1A) [5]. Matriptase is synthesized as a zymogen form comprising 855 amino acid residues and is converted to an active form by cleavage of the peptide bond between Arg614 and Val615 (Fig. 1A) [1–4]. The activation cleavage has been thought to be catalyzed by another molecule (known as transactivation) [2,6]. In addition, matriptase activation appears to be accompanied by its release from cell surface (ectodomain shedding) [7]. The activated matriptase has trypsin-like activity, and is known to cleave to activate a variety of proteins, including single-chain urokinase-type plasminogen activator (sc-uPA) [4,8–10]. These enzymatic characteristics, together with the abundant expression in surface-lining epithelial cells such as enterocytes [4], lead to the suggestion that matriptase is the key upstream regulator of the epithelial-cell turnover, including proliferation, migration, differentiation, and exfoliation.

To date, we have produced secreted variants of rat recombinant matriptase (r-matriptase) consisting of the entire extracellular domain (HL-matriptase) or of the catalytic serine protease domain (and the N-terminal spacer region) (L-matriptase) (Fig. 1A) with S-tag at their N-termini, in a Chinese hamster ovary (CHO) cell-based expression system [11,12]. In both variants, five amino-acid residues at the activation cleavage site (Thr–Lys–Gln–Ala–Arg614) were changed to those for cleavage by enterokinase (Asp–Asp–Asp–Asp–Lys) (Fig. 1A). The variants were purified as enzymatically inactive forms (pseudozymogens) but could be successfully converted to active forms by cleavage with a recombinant form of enterokinase (hereinafter called r-EK) [11,12]. The substrate specificity of HL-matriptase and L-matriptase treated with r-EK agreed with that of this protease purified from human milk [8,11,12]. In general, however, the productivity of recombinant proteins in mammalian cell-based systems is low. Indeed, the r-matriptase variants were purified from 1 l of cell culture supernatants in microgram quantities [11,12]. For further characterization of the enzymatic properties, other options to obtain larger amounts of r-matriptase were desired.

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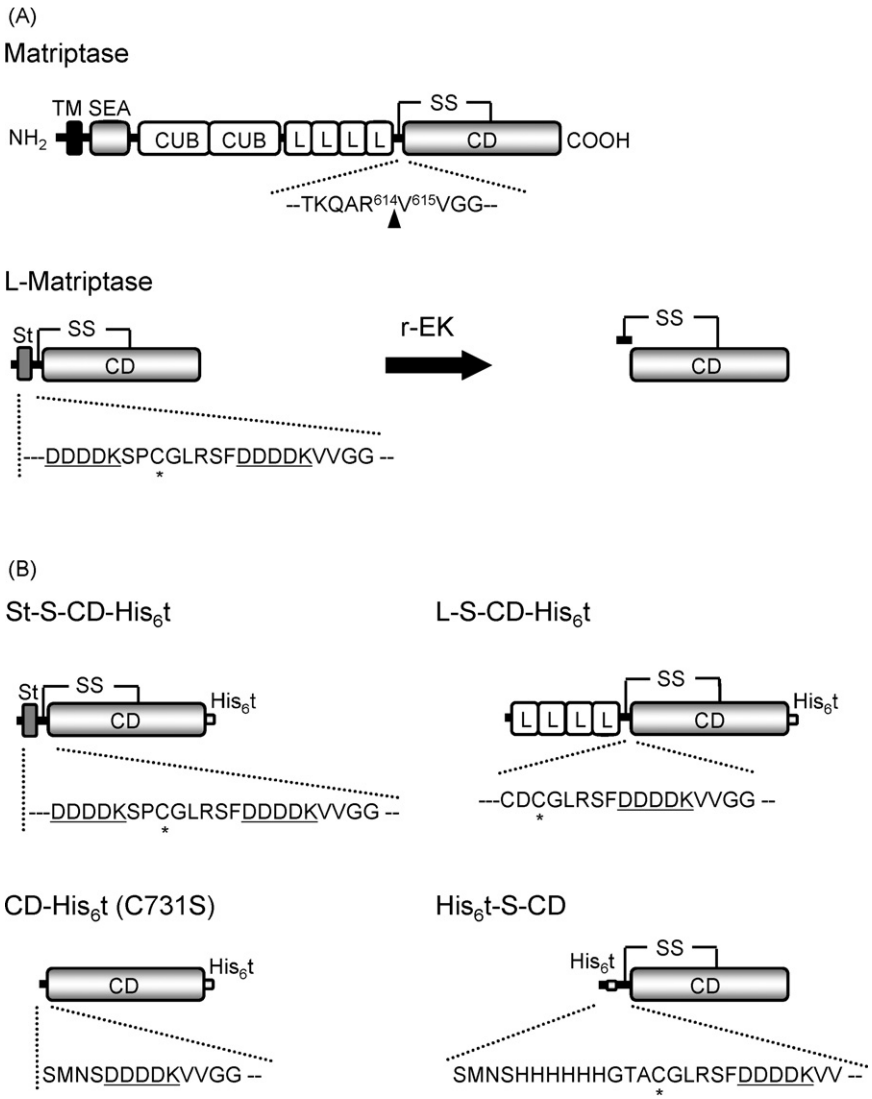

Fig. 1. Schematic illustration of the structure of rat matriptase and the expression constructs. (A) Structures of full-length rat matriptase (Matriptase) and L-matriptase. The amino acid numbering starts from the putative N-terminus of full-length matriptase. The N- and C-termini are indicated by NH_2 and COOH, respectively, in full-length matriptase. The amino acid sequence around the matriptase activation cleavage site is indicated in the single-letter code with amino acid numbering at Arg614 and Val615. The activation cleavage site is indicated by arrowhead. L-Matriptase is a secreted variant of r-matriptase in which the cytosolic, signal anchor, and stem domains (Met1–Asp603) are replaced with the human immunoglobulin κ-chain signal peptide and S-tag (ST). L-Matriptase has been produced in CHO-K1 cells [12]. L-Matriptase is converted to an active form *via* cleavage with r-EK. (B) Schematic illustration of products secreted by *P. pastoris*. In (A) and (B), the predicted disulfide linkages between two cysteine residues corresponding to Cys604 and Cys731 in full-length matriptase and r-matriptase variants except CD-His_6t (C731S) are shown as SS. Enterokinase recognition sequences (DDDDK, underlined) and their surrounding sequences are shown in all r-matriptase variants. The position corresponding to Cys604 is indicated by asterisk. Cys602 on the 4th LDL domain and Asp603 are shown in the illustration of L-S-CD-His_6t. In CD-His_6t (C731S) and His_6t-S-CD, each of putative N-terminal sequence is indicated. TM, transmembrane domain; SEA, sea-urchin sperm protein–enterokinase–agrin domain; CUB, complement factor 1R–urchin embryonic growth factor–bone morphogenetic protein domain; L, LDLRA domain; CD, catalytic domain.

In the present study, we expressed a few r-matriptase pseudozymogens in a methylotrophic yeast, *Pichia pastoris* (*P. pastoris*) expression system. The system has many advantages in high-level production of eukaryotic heterologous proteins such as genetic manipulability, quickness of growth rate, inexpensive culture cost, and the subcellular machinery for posttranslational modification of eukaryotes. We describe that the yeast enables the production of an r-matriptase pseudozymogen in milligram quantities and that the protein after treatment with r-EK exhibited activity comparable to that of activated L-matriptase.

2. Materials and methods

2.1. Materials

KM71H (a strain of *P. pastoris*), pPICZαC vector, zeocin antibiotic, and all synthetic oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). Bovine r-EK and pT7Blue-2 vector were purchased from Novagen (Madison, WI, USA). KOD_{-plus-} DNA polymerase, T4 DNA polymerase, T4 polynucleotide kinase, and a DNA ligation kit were from Toyobo (Osaka, Japan). Spectrozyme tPA® (Sp-tPA, methylsulfonyl-D-cyclohexyltyrosyl-glycyl-arginine-*p*-nitroanilide acetate) was purchased from American Diagnostica (Stanford, CA, USA). Peptidyl-4-methyl-coumaryl-7-amide (MCA) substrates were purchased from Peptide Institute (Osaka, Japan). A recombinant human sc-uPA (r-sc-uPA) was purchased from Technoclone (Vienna, Austria). Bovine trypsinogen was from Sigma (St. Louis, MO, USA). Prestained protein markers (broad range) and restriction endonucleases were purchased from New England BioLabs (Beverly, MA, USA). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Construction of expression plasmids

A plasmid (pSec-ekMT-SP1s) for expression of HL-matriptase in mammalian cells has already been constructed using pSecTag2/HygroB vector (Invitrogen) [13]. A plasmid, pPICZαC-St-S-CD-His_6t, for expression of St-S-CD-His_6 was constructed as follows: A DNA fragment was amplified by polymerase chain