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Efficient production of human goose-type lysozyme 2 in the methylotrophic yeast *Pichia pastoris*

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ABSTRACT

Infectious diseases caused by antibiotic multidrug-resistant microorganisms are major causes of morbidity and mortality in humans. Hence, there is an urgent need to search for new antimicrobial agents. Initially known as a defensive effector in the innate immunity of certain organs of the human body, human goose-type lysozyme 2 (hLysG2) has been shown to possess therapeutically useful potential against multidrug-resistant microorganisms. Developing a novel strategy for large-scale production that provides high yields of this protein with high purity, quality, and potency is critical for pharmaceutical applications. To overcome the issues related to prokaryotic expression, here we report the production of recombinant hLysG2 (rhLysG2) using the methylotrophic yeast *Pichia pastoris* as expression host. The strong inducible alcoholoxidase 1 (*AOX1*) promoter was used to drive expression of the optimized hLysG2 gene. Under the optimal expression conditions, the lytic activity of rhLysG2 reached 113 U/mL of culture supernatant in shake flask cultivation and this was increased to 2084 U/mL in fed-batch fermentation. Using chitin affinity chromatography and size-exclusion chromatography, rhLysG2 was produced with a yield of 137 mg/L, purity of > 99%, molecular weight of 21,504.6 Da, and specific activity of 13,500 U/mg. *In vitro* assays indicated that rhLysG2 possessed muramidase activity, isopeptidase activity, and free radical scavenging activity. This report describes an efficient strategy for the production of biologically active rhLysG2 in *P. pastoris* on a large scale with a high yield, which provides a solid foundation for possible future pharmaceutical applications.

1. Introduction

Lysozymes are ubiquitous hydrolases which exist in numerous phylogenetically diverse organisms, including animals, plants, fungi, bacteria and bacteriophages. According to differences in amino acid composition, molecular structure and enzymatic properties, lysozymes are categorized into six types: goose-type (g-type), chicken-type (c-type), invertebrate-type (i-type), phage, bacterial and plant lysozymes (Bachali et al., 2004; Hikima et al., 2003; Jollès et al., 1996). The innate immune system is recognized as an effective first line of defense to protect organisms against invading pathogens. Many studies have indicated that different types of lysozymes, such as c-type and g-type, function as important defense effectors in the innate immune system of animals by hydrolyzing glycosidic bonds in the bacterial cell wall peptidoglycan, resulting in bacteriolysis (Jollès and Jollès, 1984; Callewaert and Michiels, 2010).

The g-type lysozyme was firstly found in Embden goose egg whites and was recognized as a bactericidal enzyme (Canfield and McMurry, 1967). Subsequently, it was identified in other vertebrates, including mammals (MGCP Team, 2002), birds (Simpson et al., 1980; Nakano and Graf, 1991; Maehashi et al., 2012), fish (Myrnes et al., 2013; Liu et al., 2016; Nilojan et al., 2017), and invertebrates, such as abalone (Bathige et al., 2013) and scallops (Li et al., 2013). G-type and c-type lysozymes were encoded by two different genes and have significant differences in many aspects (Arnheim et al., 1973; Weaver et al., 1985). C-type lysozyme contains two essential catalytic sites, while g-type lysozyme

Abbreviations: hLysG2, human goose-type lysozyme 2; AOX1, alcoholoxidase 1; MD, minimal dextrose; YPD, yeast extract peptone dextrose; BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; PCR, polymerase chain reaction; RT-PCR, real-time fluorescent quantitative PCR; *gapdh*, glyceraldehyde 3-phosphate dehydrogenase gene; WCW, wet cell weight; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OD, optical density; DO, dissolved oxygen; BSA, bovine serum albumin; PBS, phosphate buffered saline; RP-HPLC, reverse-phase high performance liquid chromatography; LC–MS, liquid chromatography mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; L-γ-Glu-pNA, L-γ-glutamine-p-nitroanilide; pNA, p-nitroanilide; MOPS, 3-(N-morpholino) propanesulfonic acid; DPPH, 1, 1 diphenyl-2-picryl hydrazyl

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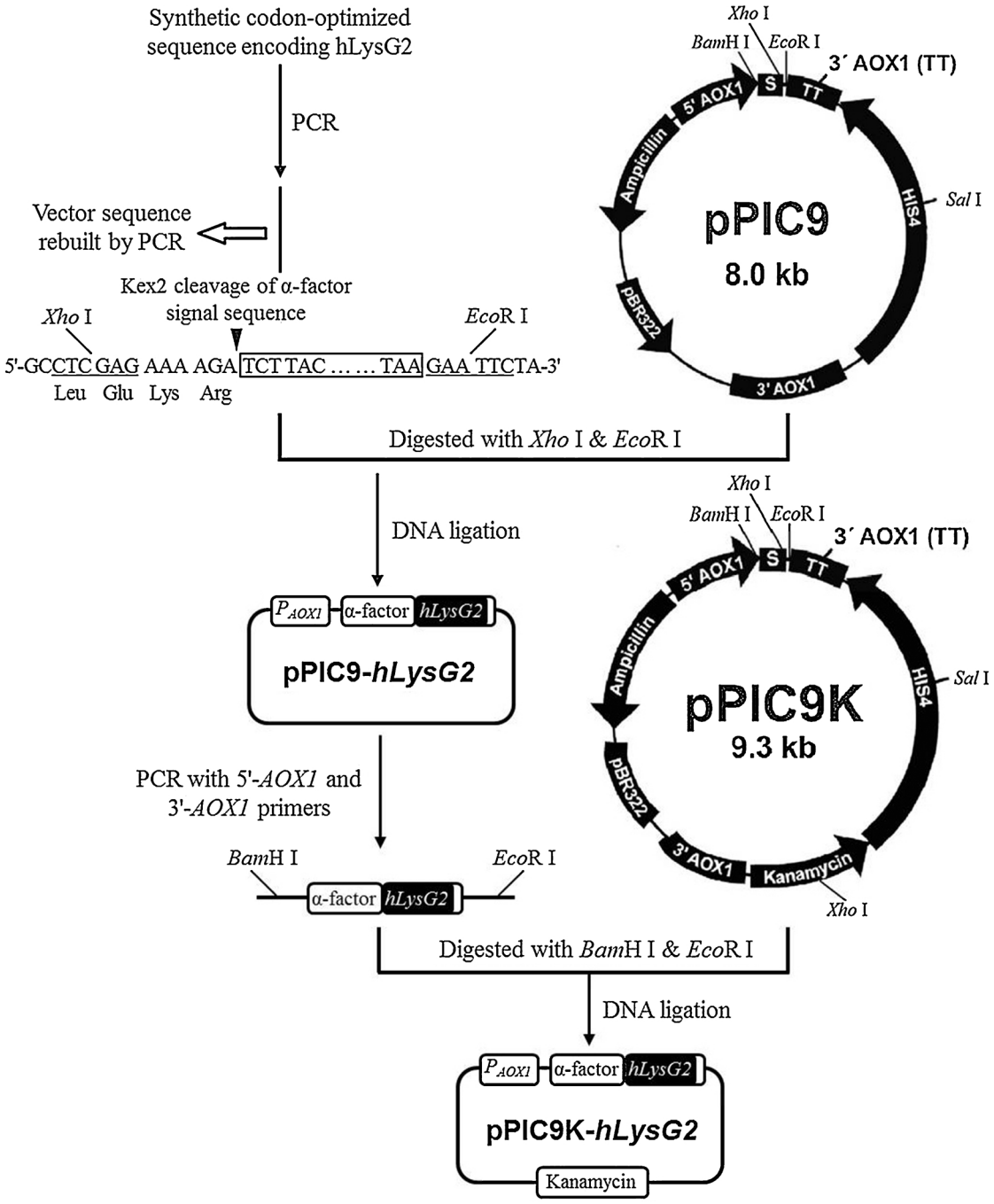

Fig. 1. Construction of the pPIC9K-*hLysG2* expression plasmid.

possesses three essential catalytic sites. No signal peptide is found in some g-type lysozymes from fish, which suggests that these kinds of g-type lysozymes may be secreted from tissues or cells by a non-classical pathway (Fu et al., 2013; Wei et al., 2014). The human g-type lysozyme subfamily consists of two members, human g-type lysozymes 1 and human g-type lysozyme 2 (hLysG2), and only the latter conserves several key molecular features for catalytic activity, including retention of putative substrate binding and three essential catalytic residues (Irwin and Gong, 2003; Irwin, 2014).

Recently, the incidence of multidrug resistance in pathogenic and opportunistic bacteria has been increasingly documented (Jones et al., 2004). The reduced susceptibility of multidrug-resistant bacteria to available antibiotics is continuously increasing. In the previous study, we confirmed the constitutive expression of hLysG2 in the eye and testis in humans and its presence in tears. The recombinant expression of hLysG2 was achieved in the shake flask using the methylotrophic yeast *P. pastoris* as an expression system. The significant role of rhLysG2 in inhibiting the growth of Gram-positive bacteria was demonstrated, suggesting that hLysG2 may be an important defense effector protecting the human eye against infection (Huang et al., 2011). As it has an antibacterial mechanism completely dissimilar to conventional antibiotics, hLysG2 has a great potential as an antimicrobial agent for use in the treatment of infectious diseases caused by multidrug-resistant microorganisms. As far as we know, the production of any kind of g-type lysozyme in prokaryotic or eukaryotic hosts on a large scale has not been reported. However, to generate larger and safer stocks of hLysG2 for pharmaceutical applications, it is desirable to establish an efficient production procedure involving the intensive culture of genetically-