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Full Length Research Paper

Improved secretory production of calf prochymosin by codon optimization and disruption of *PMR1* in *Kluyveromyces lactis* GG799

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Chymosin as an important industrial enzyme used widely in cheese manufacture. In our preliminary study, low yields (80 U mL^{-1}) were obtained when *Kluyveromyces lactis* GG799 was used to express chymosin. We investigated whether this poor secretion could be improved by codon optimization and disruption of *PMR1* gene in *K. lactis* GG799. Our study shows that combination of codon optimization and disruption of the *PMR1* gene resulted in secretion level of chymosin that was increased 10-fold. Our results show that codon optimization and disruption of the *PMR1* gene synergistically stimulate the secretion of calf prochymosin in *K. lactis* GG799.

Key words: *Kluyveromyces lactis*, codon optimization, *PMR1*, prochymosin.

INTRODUCTION

Chymosin (EC 3.4.23.4) is used extensively in cheese production because it specifically cleaves *kappa*-casein at the Phe^{105}-Met^{106} bond, with low proteolytic activity (Mohanty et al., 1999). With an increase in cheese production, limited availability of calf stomachs and ethical issues concerning animal slaughter, there is a necessity to find alternatives to calf chymosin (Ashwani et al., 2006). With developments in recombinant DNA technology, recombinant chymosins are now widely used for cheese manufacture in many countries with excellent results (Bansal et al., 2009). Many studies have focused on increasing the yield of recombinant chymosin (Van den Berg et al., 1990; Harmsen et al., 1996, 2002; Valkonen et al., 2003; Hans et al., 2006).

Kluyveromyces lactis has many advantages over other expression hosts for commercial production of enzymes. It is a GRAS (generally recognized as safe) organism approved by the US Food and Drug Administration (Van den Berg et al., 1990; Van Ooyen et al., 2006; Bo et al., 2009). In our preliminary study, codon optimization or disrupting the *PMR1* gene improved chymosin production in *K. lactis* GG799 (Feng et al., 2010, 2011). Shi-Hwei et al. (2005) improved the secretion level of glucoamylase in *Pichia pastoris* by the combination of genetic manipulations. However, the enhanced secretion of recombinant chymosin by codon optimization and disruption of the *PMR1* gene has not been reported in *K. lactis*. Therefore, in this study, we attempted to increase further the expression of calf chymosin through combination of codon optimization and disruption of the *PMR1* in *K. lactis* GG799.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The *PMR1*-deficient mutant of *K. lactis* GG799 [*K. lactis* GG799 (*pmr1*Δ)] was constructed in our preliminary experiments (Feng et al., 2011). The recombinant plasmid, pKLAC1-O-prochymosin (including the optimized chymosin gene), was constructed in our preliminary experiments (Feng et al., 2010). The *K. lactis* GG799(*pmr1*Δ) cells were grown in YPD-$CaCl_2$ medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose and 1.11 g L^{-1} $CaCl_2$) at 28 to 30°C.

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Table 1. Microbial strains used in the present study and its biomass at culture termination.

Host	Gene	Recombinant strain	Reference	Biomass (g L^{-1})
K. lactis GG799	Native chymosin	KL1	(Feng et al., 2010)	12.07 ± 0.67
	Optimized chymosin	KL2	(Feng et al., 2010)	12.09 ± 0.53
K. lactis GG799 (*pmr1*Δ)	Native chymosin	KL3	(Feng et al., 2011)	11.52 ± 0.82
	Optimized chymosin	KL4	This study	12.09 ± 0.65

Transformation of *K. lactis* GG799 (*pmr1*Δ) and selection of multi-copy integrants

The recombinant plasmid, pKLAC1-O-prochymosin, was digested with *Sac*II to linearize plasmid and transformed into *K. lactis* GG799 (*pmr1*Δ). Transformants containing the desired insert were selected on yeast carbon base (YCB) agar medium containing 5 mmol L^{-1} acetamide and 10 mmol L^{-1} $CaCl_2$. Multiply integrated cells were identified by colony PCR. The manufacturer's instructions were used for the transformation of *K. lactis* GG799 and the selection of multi-copy integrants (*K. lactis* Protein Expression Kit Instruction Manual, New England Biolabs, Inc., Ipswich, United States).

Protein expression and assay of chymosin activity

The transformants were further screened for their ability to secrete recombinant chymosin in YPD-$CaCl_2$ medium. For expression of the recombinant protein, colonies were grown in 150 ml shaking flasks (250 rpm) containing 30 ml of YPD medium at 30°C for 4 to 5 days. Enzyme activity of the culture supernatant was assayed every 6 h during fermentation.

Culture supernatants were acidified to pH 2 with 1 mol L^{-1} H_2SO_4, incubated for 2 h at room temperature, and then neutralized to pH 6 with 2 mol L^{-1} Tris base. An appropriately diluted sample was added to 5 ml of a suspension of 12% non-fat dry milk in 10 mmol L^{-1} $CaCl_2$ and incubated at 37°C until a clot formed. One unit of chymosin activity is defined as the amount of active chymosin required to produce a clot in 40 min under these conditions (Van den Berg et al., 1990).

RESULTS AND DISCUSSION

Potential bottlenecks for protein secretion include the codon usage of the expressed gene, the efficiency and strength of promoters, translation signals, signal peptides, processing and folding in the endoplasmic reticulum (ER) and Golgi, and environmental factors of extracellular secretion (Diethard et al., 2004). Many of the problems encountered in protein expression can be overcome by proper consideration of the influencing factors. To examine whether the disruption of the *PMR1* and codon optimization of the calf prochymosin gene affected enzyme secretion efficiency, the *K. lactis* GG799 (*pmr1*Δ) was transformed with the plasmid, pKLAC1-O-prochymosin, to obtain the strain, KL4.

In our preliminary experiments, the strain KL1, KL2 and KL3 were constructed (Table 1). The strain KL1, KL2, KL3 and KL4 were fermented under the same conditions. All the media were supplemented with 10 mmol L^{-1} calcium chloride. During shake-flask fermentation, the strain KL4 expressed substantially higher enzymatic activity of recombinant calf chymosin than the strain KL1, KL2 and KL3 as control, at various fermentation stages (Figure 1).

The enzymic activity of recombinant calf chymosin from KL4 reached the highest expression level of 860 U mL^{-1}. At the same time, the enzymic activity of recombinant calf chymosin from KL1, KL2 and KL3 was 80 U mL^{-1}, 575 U mL^{-1} and 496 U mL^{-1}, respectively. In the case of biomass, the dry cell weight of KL1, KL2, KL3 and KL4 showed no substantial differences (Table 1).

In our study, the recombinant strains, KL1, KL2, KL3 and KL4 were fermented under the same conditions. As far as the expression of native chymosin gene and the optimized chymosin gene in wild type *K. lactis* GG799 is concerned, the expression level of the optimized chymosin gene based on preferred codons in *K. lactis* GG799 increased 7-fold (KL1 compared with KL2). As far as expression of the native chymosin gene in wild type *K. lactis* GG799 and *K. lactis* GG799 (*pmr1*Δ) is concerned, the *PMR1* mutant strain secretion of chymosin was increased by 6-fold over that of the wild type (KL1 compared with KL3). As far as expression of the optimized chymosin gene in *K. lactis* GG799 (*pmr1*Δ) is concerned, codon optimization and *PMR1* disruption synergistically increase the secretion of chymosin, with an increase in the secretion level of chymosin of 10-fold (KL1 compared with KL4). Our investigation shows that cells expressing chymosin at a high level secrete considerably more of the protein produced than expected on the basis of the effect of either mutation by itself, indicating that *PMR1* disruption and codon optimization act synergistically. Shi-Hwei et al. (2005) increased the production of glucoamylase in *P. pastoris* employing combination of modified signal peptide, increased copy number of the gene and over expression of *SEC4*. Harmsen et al. (1996) stimulated secretion of calf prochymosin by over expression of the binding protein and disruption of the *PMR1* gene in *Saccharomyces cerevisiae*. The present results are similar to aforementioned studies.

Many genetic and environmental factors can influence the efficiency of recombinant protein production in gene expression systems. Since these factors are cross-regulated, it is of importance to optimize the variables involved in the expression system of choice to achieve the highest yields. There were some reports about increasing the production of chymosin by codon optimization or *PMR1* inactivation in other expression hosts (Harmsen et

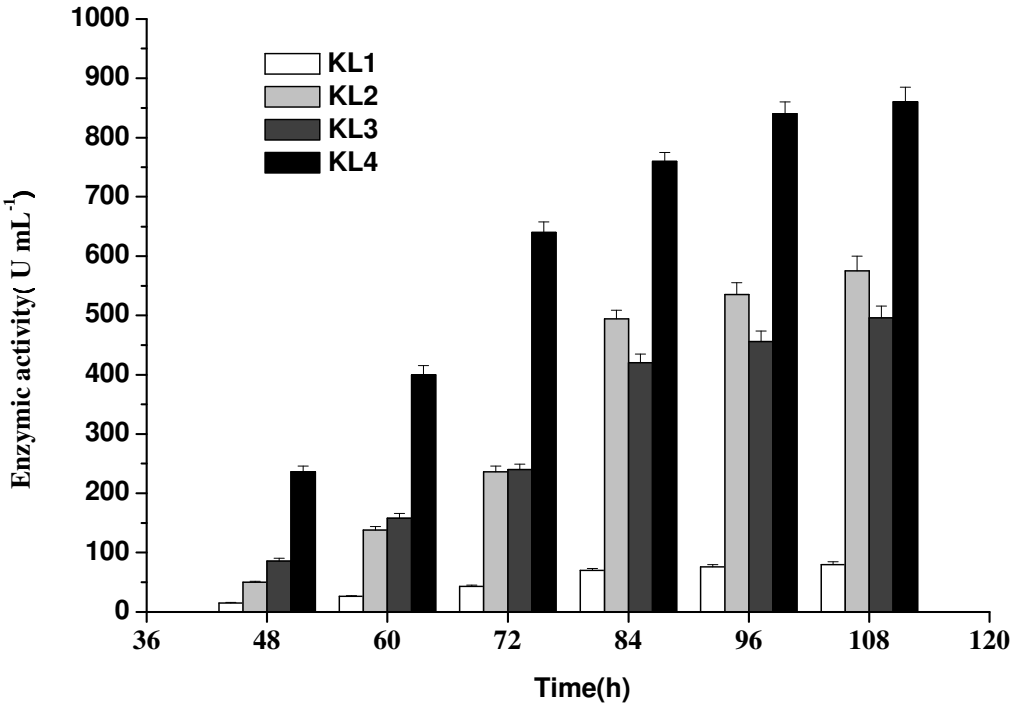

Figure 1. Comparison of enzymic activity of recombinant chymosin produced by KL1, KL2, KL3 and KL4 at various culture stages.

al., 1996; Cardoza et al., 2003; Tokuoka et al., 2008). There were only a few examples of the improvement of chymosin production by genetic manipulation in *K. lactis*. However, the enhanced secretion of recombinant chymosin by codon optimization and disruption of the *PMR1* gene has not been reported in *K. lactis*. In this paper, we report a 10-fold enhancement of the secretory production of chymosin in *K. lactis* GG799 employing a combination codon optimization and disruption of the *PMR1* gene. Genetic manipulation of the expression host, such as that described, may improve chymosin production. This improved secretory production of calf chymosin in *K. lactis* has potential applications for industrial chymosin production. Such genetic manipulation will be useful for the expression of other mammalian genes in yeast systems and may be used to improve existing expression systems for this gene.

Besides codon optimization and disruption of *PMR1*, there were other possible ways too, to enhance the heterologous protein expression in *K. lactis*. Raimondi et al. (2010) increased the production of human serum albumin and glucoamylase by *KlSOD1* gene over expression in *K. lactis*. Over expression of GDP-mannose pyrophosphorylase, *KlPSa1p* gene of *K. lactis* has also been found to enhance the heterologous protein secretion (Uccelletti et al., 2005). Glucose repression defective mutant of *K. lactis* is also found to enhance the production of heterologous proteins (Donnini et al., 2004). The feasibility of improving chymosin secretion in *K. lactis* GG799 depends on whether the several steps of protein processing are close to their limits. In next research, we will investigate whether chymosin production is also enhanced by the aforementioned methods in *K. lactis*.

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