Note

Improved Direct Expression of Prochymosin cDNA through Changing the SD-ATG Codon Length

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Calf prochymosin is a precursor of the milk-clotting protease, chymosin, which is essential for cheese manufacturing. The NH₂-terminal peptide of 42 amino acids is autocatalytically removed from prochymosin at acidic pH to form active chymosin. We have previously reported the construction of hybrid plasmids which allow expression of prochymosin cDNA fused to *lacZ* or *trpE*, L under the control of the *lac* or *trp* promoter, respectively.^{1,2)} *Escherichia coli* harboring these plasmids produced large amounts of prochymosin as inclusion bodies in the cells, which was effectively converted to chymosin by extraction with urea followed by alkaline dialysis.³⁾ Another plasmid that allowed direct expression of N-terminal methionyl Met-prochymosin under the control of the *trp* promoter was also constructed, however, it gave a far lower yield of prochymosin, probably due to the greater distance between the SD sequence and the ATG codon.²⁾ In order to improve the efficiency of direct expression, we derived plasmids with various SD-ATG lengths and examined their expression efficiencies.

pCR701²⁾ is a plasmid containing the *trp* promoter and the cDNA sequence for Met-prochymosin with an SD-ATG length of 14 bp. The scheme for changing the SD-ATG length in pCR701 is shown in Fig. 1. Successive *Hind*III cleavage, repair with DNA polymerase I and ligation with T4 DNA ligase gave pCR711 with an SD-ATG length of 18 bp. Treatment of the *Hind*III-fragments with nuclease S1 followed by ligation gave pCR712 and pCR713 which had reduced SD-ATG lengths of 8 and 6 bp, respectively. pCR714 with a different 6 bp sequence was derived from pCR712 by *Cla*I digestion, limited repair and ligation. The construction of all these plasmids was confirmed by sequencing analysis according to the method of Maxam and Gilbert.⁴⁾

These derivative plasmids were introduced into *E. coli* C600 rk⁻ mk⁻ by the procedure of Norgard *et al.*⁵⁾ pCR301 carrying the *lacUV5* promoter and a fused prochymosin cDNA in which the NH₂-terminal 4 amino acids of prochymosin were replaced with the NH₂-terminal 10 amino acids of β -galactosidase¹⁾, and pCR501 carrying the *trp* promoter and a similar fused prochymosin cDNA with the *trpE* gene²⁾ were also examined as controls. Seed cultures of *E. coli* carrying these plasmids were performed in L-broth containing 50 μ g/ml ampicillin at 37°C. 10 ml of each culture of *E. coli* C600 except for the pCR301 transformant was inoculated at the mid-log phase into 100 ml of fresh M9 medium containing 2% casamino acids, 0.2% glucose and 50 μ g/ml ampicillin. After 1 hr cultivation, 15 μ g/ml β -indolylacrylic acid was added. 10 ml of the seed culture of *E. coli* GM1 (pCR301) was transferred to 100 ml of L-broth containing 50 μ g/ml ampicillin and 2 mM isopropyl- β -D-thiogalactopyranoside. After another 5 hr cultivation, the cells were harvested and the total cellular proteins were determined by SDS-gel electrophoresis, and detection of the prochymosin polypeptide was performed by immunoblotting with a Bio-Rad immunoblotting kit.

Among the derivatives of pCR701, pCR712 with a 8 bp SD-ATG length showed enhanced production of prochymosin, while pCR711, pCR713 and pCR714 showed lower yields than that of the original pCR701 (Fig. 2). The amounts of prochymosin in the extracts were determined from the milk-clotting activity which was measured after extraction with 8 M urea followed by alkaline dialysis,³⁾ and the enzyme-linked immunosorbent assay using β -

FIG. 1. Construction of Derivative Plasmids of pCR701.

FIG. 2. Enzyme Immuno-blotting Detection of Prochymosin Produced by *E. coli* Harboring Various Plasmids.

SDS-gel electrophoresis was carried out as described previously.¹⁾ 1 and 9, authentic prochymosin; 2, pCR301; 3, pCR501; 4, pCR701; 5, pCR711; 6, pCR712; 7, pCR713; 8, pCR714. Prochymosin was produced by *E. coli* CM1 (2) or *E. coli* C600 (3~8) as the host.

galactosidase-linked anti-prochymosin antibodies prepared by the method of Hamaguchi *et al.*⁶⁾ Prochymosin production by pCR712 was better than that by pCR701 or pCR501 (Table 1). The appropriate SD-ATG length in pCR712, that is similar to the natural 7 bp length in the *trp* promoter, seems to be effective for improving the prochymosin productivity. It is of interest that pCR713 and pCR714, which have identical SD-ATG lengths of 6 bp, showed a marked difference in prochymosin production. It is evident that not only the length but also the base sequence between SD-ATG affects the translation efficiency.

Table I also shows that the prochymosin polypeptide detected by the immunological assay was almost completely converted to active chymosin. However, we have observed that quantitative conversion of bacterially synthesized prochymosin to the active form is difficult with *E. coli* HB101 as the host. The lower concentrations of

TABLE I. QUANTITATIVE ESTIMATION OF PROCHYMOSIN PRODUCED BY *E. coli*

Plasmids	Prochymosin ($\mu\text{g/ml}$) ^a (Clotting activity)	Prochymosin ($\mu\text{g/ml}$) ^b (ELISA)
pCR301	13.6	14.0
pCR501	7.7	7.5
pCR701	2.9	3.2
pCR711	0.4	0.8
pCR712	9.5	9.8
pCR713	ND	0.3
pCR714	0.5	0.9

^a Estimated from the milk-clotting activity after acid treatment in comparison with the activity of authentic prochymosin.

^b Estimated from the standard curve for the ELISA (enzyme-linked immunosorbent assay) using 8M urea treated authentic prochymosin.

prochymosin in the extracts of the *E. coli* C600 strains might be the reason for the quantitative conversion to the active form.

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