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Efficient secretory production of alkaline phosphatase by high cell density culture of recombinant *Escherichia coli* using the *Bacillus* sp. endoxylanase signal sequence

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Abstract New secretion vectors containing the *Bacillus* sp. endoxylanase signal sequence were constructed for the secretory production of recombinant proteins in *Escherichia coli*. The *E. coli* alkaline phosphatase structural gene fused to the endoxylanase signal sequence was expressed from the *trc* promoter in various *E. coli* strains by induction with IPTG. Among those tested, *E. coli* HB101 showed the highest efficiency of secretion (up to 25.3% of total proteins). When cells were induced with 1 mM IPTG, most of the secreted alkaline phosphatase formed inclusion bodies in the periplasm. However, alkaline phosphatase could be produced as a soluble form without reduction of expression level by inducing with less (0.01 mM) IPTG, and greater than 90% of alkaline phosphatase could be recovered from the periplasm by the simple osmotic shock method. Fed-batch cultures were carried out to examine the possibility of secretory protein production at high cell density. Up to 5.2 g/l soluble alkaline phosphatase could be produced in the periplasm by the pH-stat fed-batch cultivation of *E. coli* HB101 harboring pTrcS1PhoA. These results demonstrate the possibility of efficient secretory production of recombinant proteins in *E. coli* by high cell density cultivation.

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Introduction

Escherichia coli has been the workhorse for the production of recombinant proteins. Recombinant proteins can be synthesized in *E. coli* and guided to four different locations: the cytoplasm, the periplasmic space, the inner or outer membrane, and the extracellular medium. Proteins found in the outer membrane or periplasmic space are synthesized in cytoplasm as premature (unprocessed) proteins. These premature proteins have specific hydrophobic N-terminal sequences (signal sequences) which are cleaved by signal peptidases during the translocation process (Pugsley 1993). Secretion of various proteins across the cytoplasmic membrane can be achieved by fusing an appropriate signal sequence to the N-terminus of the target gene.

Secretory production of proteins has several advantages as follows (reviewed in Makrides 1996). First, the N-terminal amino acid residue of the secreted product can be identical to the natural gene product as a result of the cleavage of the signal peptide by a specific signal peptidase. Second, protease activities are much less in the periplasmic space than in the cytoplasm. Third, purification of recombinant proteins is much simpler due to the presence of fewer contaminating proteins in the periplasm. Fourth, correct formation of disulfide bonds can be facilitated because the periplasmic space provides an oxidative environment (Hockney 1994; Makrides 1996). For the efficient secretion of recombinant proteins in *E. coli*, various signal sequences including OmpA (Barthelemy et al. 1993), LamB (Klein et al. 1992), OmpF (Shibui et al. 1991) and PelB (Lucic et al. 1998) were examined. However, they did not always ensure successful secretion of recombinant proteins (Klein et al. 1992; Lucic et al. 1998), for several reasons that included autolytic activities of weakened outer membrane, low product levels, incomplete processing, and different characteristics of the proteins to be secreted in *E. coli*. Furthermore, secretory production of recombinant proteins

by high-cell-density culture has received relatively little attention.

In this report, a novel endoxylanase signal sequence from *Bacillus* sp. was employed for the efficient secretion of a model protein alkaline phosphatase. Secretory production of alkaline phosphatase at high cell density was also examined.

Materials and methods

Bacterial strains, plasmids, and DNA manipulation

The bacterial strains and plasmids used in this study are summarized in Table 1. *E. coli* XL1-Blue was used as a host strain for the construction of recombinant plasmids. Various *E. coli* strains including HB101, XL1-Blue, BL21(DE3), and KS272 were examined for the secretory production of alkaline phosphatase. In particular, *E. coli* KS272 was included because it is an alkaline phosphatase negative (*ΔphoA*) mutant strain (Meerman and Georgiou 1994). Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). Polymerase chain reaction (PCR) was performed with a PCR Thermal Cycler MP TP3000 (Takara Shuzo Co., Japan) using a High Fidelity PCR System (Boehringer Mannheim, Mannheim, Germany). All DNA manipulations were carried out as described by Sambrook et al. (1989).

Culture conditions

Flask cultures were carried out in 250-ml flasks containing 50 ml LB medium (per liter: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g) supplemented with 50 mg/l ampicillin in a shaking incubator at 37 °C and 250 rpm. Cells were induced at the OD_{600} of 0.7 by adding of 0.01, 0.1 or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Chemical Co., St. Louis, Mo.) and cell growth was continued for 5 h. Fed-batch cultures were carried out in R/2 medium (pH 6.8) (Park et al. 1999), which contains per liter: $(NH_4)_2HPO_4$, 2 g; KH_2PO_4, 6.75 g; citric acid, 0.85 g; $MgSO_4 \cdot 7H_2O$, 0.7 g; trace metal solution (per liter of 5 M HCl: $FeSO_4 \cdot 7H_2O$, 10 g; $ZnSO_4 \cdot 7H_2O$, 2.25 g; $CuSO_4 \cdot 5H_2O$, 1 g; $MnSO_4 \cdot 5H_2O$, 0.5 g; $Na_2B_4O_7 \cdot 10H_2O$, 0.23 g; $CaCl_2 \cdot 2H_2O$, 2 g; $(NH_4)_6MO_7 \cdot O_{24}$, 0.1 g), 5 ml. Fed-batch cultures were carried out in a 6.6-l jar fermenter (Bioflo 3000, New Brunswick Scientific Co., Edison, NJ) containing 2 l R/2 medium supplemented with 10 g/l glucose, 2 g/l yeast extract, and 100 mg/l ampicillin. Seed culture (200 ml) was prepared in the same medium. Culture pH was controlled at 6.8 by the addition of 28% (v/v) ammonia water. The dissolved oxygen concentration (DO) was controlled at 40% of air saturation by automatically increasing the agitation speed up to 1,000 rpm and by changing the pure oxygen percentage. The nutrient feeding solution used for the fed-batch culture contains per liter: glucose, 500 g; yeast extract, 100 g; $MgSO_4 \cdot 7H_2O$, 15 g. Feeding solution was added by the pH-stat feeding strategy (Lee and Chang 1994; Lee 1996). When the pH rose to a value greater than its set point (6.8) by 0.05 due to the depletion of glucose, the appropriate volume of feeding solution was automatically added to increase the glucose concentration in the culture broth to 0.5 g/l. Expression of alkaline phosphatase gene was induced by adding IPTG to give a final concentration of 1 mM at the time of induction. Cell dry weight was determined as described previously (Lee and Chang 1994). *E. coli* HB101 harboring pTrcS1PhoA was cultured at 37 °C, while *E. coli* KS272 was cultured at 30 °C because it cannot be grown to high cell density at high temperature (Park et al. 1999).

Cell fractionation

Cells from 1 ml culture broth were harvested by centrifugation at 10,000 *g* for 5 min at 4 °C. The periplasmic fractions were prepared by the modified osmotic shock method reported by Park and Lee (1998). The inclusion body and soluble protein fractions were prepared as follows. The harvested cells were centrifuged at 3,500 *g* for 5 min at 4 °C and the pellet was washed with 0.5 ml TE buffer (pH 8.0), followed by centrifugation at 3,500 *g* for 5 min at 4 °C. The pellet was resuspended in 0.2 ml TE buffer (pH 8.0), and the solution was sonicated thoroughly to disrupt cells. The inclusion bodies from the sample were isolated by centrifugation at 17,000 *g* for 30 min at 4 °C and were resuspended in 0.2 ml TE buffer. The supernatant was recovered as the soluble protein fraction.

Determination of N-terminal sequence of secreted protein

The periplasmic fraction was loaded on the SDS-PAGE and the protein on the gel was electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, Calif.). The alkaline phosphatase band on the PVDF membrane was excised and sequenced on an Applied Biosystem gas phase sequencer, Model 476A (Applied Biosystem Inc. Foster, Calif.).

Analytical methods

SDS-PAGE was carried out on 12% polyacrylamide separation gel containing 0.1% (w/v) SDS as described by Laemmli (1970).

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
XL1-Blue	*SupE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F′(proAB*$^+$ lacI^q *lacZΔM15 Tn10(tet^r))*	Stratagene[a]
BL21(DE3)	F$^-$ *ompT hsdS$_B$ (r_B^- m_B^-) gal dcm (DE3)*	Novagen[b]
MC4100	F$^-$ *araD139 Δ(argF-lac)U169 rpsL150(str^r) relA1 flbB5301 deoC1 ptsF25 rbsR*	Lab stock
KS272	F$^-$ *ΔlacX74 galE galK thi rpsL (strA) ΔphoA(pvuII)*	Meerman and Georgiou 1994
HB101	F– *hsdS20 (r_k^- m_k^-) recA13 ara-14 proA2 lacY1 galK2 rpsL20(str) xyl-5 mtl-1 SupE44λ*	Lab stock
Plasmids		
pKJX4	4.3 kb, Ap^r	Jeong et al. 1998
pTrc99A	4.2 kb, Ap^r, *trc* promoter	Pharmacia[c]
pJS101	4.9 kb, endoxylanase, Ap^r, *trc* promoter	This study
pJS101ΔP	4.9 kb, endoxylanase, Ap^r, *trc* promoter	This study
pTrcS1PhoA	5.6 kb, endoxylanase signal sequence, alkaline phosphatase, Ap^r, *trc* promoter	This study

[a] Stratagene Cloning Systems, La Jolla, Calif.
[b] Novagen, Madison, Wis.
[c] Pharmacia Biotech, Uppsala, Sweden

Proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad) and the protein bands on the SDS-PAGE gel were quantified by GS710 Calibrated Imaging Densitometer (Bio-Rad). Alkaline phosphatase activities were determined as described previously (Brickman and Beckwith 1975). All experiments were carried out in triplicate. The alkaline phosphatase activity was calculated by the following formula as reported previously (Brickman and Beckwith 1975):

$$\text{Activity of alkaline phosphatase (unit)} = 1,000 \times \frac{\text{OD}_{420} - 1.75 \times \text{OD}_{550}}{\text{time} \times \text{OD}_{600}(\text{of culture})} \times \text{dilution factor}$$

Results

Construction of secretion vectors containing endoxylanase signal sequence

To clone the endoxylanase gene, PCR was performed using pKJX4 (Jeong et al. 1998) as a template. The forward primer 5′-GCTCAGCCGGTCTCCCATGTTTAAGTTTAAAAAGAA-3′ was designed to contain a *Bsa*I site (underlined). The reverse primer 5′-GCGGATCCGAGCTGTTACCACACAGTTAC-3′ was designed to contain a *Bam*HI site (underlined). The PCR product (650 bp) was partially digested with *Bsa*I and fully digested with *Bam*HI, and was ligated into the *Nco*I and *Bam*HI sites of pTrc99A, which contains a strong inducible *trc* promoter. In this construct (pJS101), the signal sequence of endoxylanase and mature gene of endoxylanase was connected by *Pst*I site. However, pJS101 contains another *Pst*I site within the multiple cloning site. For the deletion of *Pst*I site in the multiple cloning site, the plasmid was digested with *Xba*I and *Hin*dIII, made blunt by using Klenow enzyme, and was self-ligated. In this way, the final secretion vector, pJS101ΔP, was constructed as shown in Fig. 1A. This plasmid allows convenient cloning of genes encoding foreign protein without changing the signal sequence. The gene encoding the mature form of alkaline phosphatase (*phoA*) without its signal sequence was amplified by PCR from the chromosomal DNA of *E. coli* W3110. The forward primer 5′-GGACTGCAGCACGGACACCAGAAATGCCTGTT-3′ was designed to contain a *Pst*I site (underlined). The reverse primer 5′-GCGGGATCCTTATT ATTTCAGCCCCAGAGCCGG-3′ was designed to contain a *Bam*HI site (underlined). The PCR product (1,360 bp) was digested with *Pst*I and *Bam*HI and then cloned into pJS101ΔP (Fig. 1B). In this construct (pTrcS1PhoA), the alkaline phosphatase gene connected to the endoxylanase signal sequence can be expressed under the inducible *trc* promoter.

Secretion of endoxylanase and alkaline phosphatase in *E. coli*

To examine whether the endoxylanase signal sequence can be used for the secretory protein production in *E. coli*, three *E. coli* strains (XL1-Blue, HB101, and MC4100) harboring pJS101ΔP (Fig. 1A) were cultured at 37 °C and were induced with 1 mM IPTG at the OD_{600} of 0.7. In each *E. coli* strain, endoxylanase was successfully secreted into the periplasm of *E. coli* and the contents of secreted endoxylanase in *E. coli* XL1-Blue, HB101, and MC4100 were 11.3%, 9.4%, and 10.5% of total proteins, respectively. Therefore, it was reasoned that the *Bacillus* sp. endoxylanase signal sequence may be used to direct other proteins to the periplasm of *E. coli*.

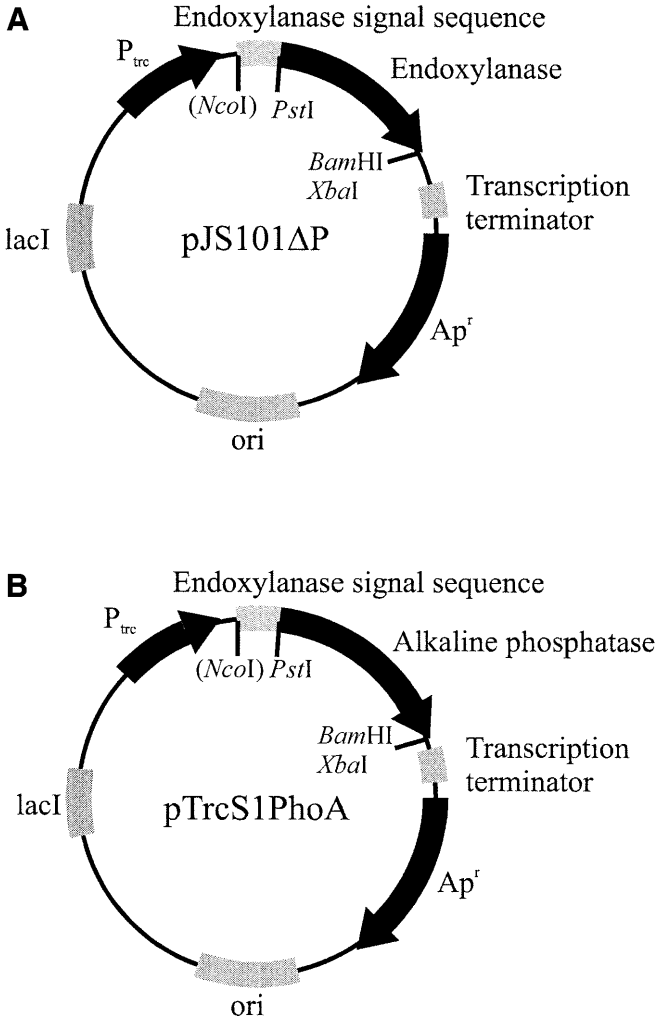

Fig. 1A, B Structures of plasmids: **A** pJS101ΔP, **B** pTrcS1PhoA

To examine whether other proteins can also be secreted efficiently using the endoxylanase signal sequence, alkaline phosphatase was used as a target protein. Alkaline phosphatase is a good model protein for studying protein secretion because it is active only after it crosses the membrane into periplasm (Derman and Beckwith, 1991). Several *E. coli* strains harboring pTrcS1PhoA, including HB101, BL21(DE3), and XL1-Blue, were cultured at 37 °C and induced with 1 mM IPTG at the OD_{600} of 0.7. Alkaline phosphatase was successfully secreted in all strains, and its content was 24.2% of total

proteins in XL1-Blue, 25.3% of total proteins in HB101, and 22.4% in BL21(DE3). However, we observed that some recombinant XL1-Blue and BL21(DE3) harboring pTrcS1PhoA was lysed after induction.

Alkaline phosphatase is a secreted protein, encoded by a single structural gene (*phoA*), which is synthesized under phosphate starvation during cultivation. We also examined whether alkaline phosphatase is secreted in *E. coli* KS272 strain, which is alkaline phosphatase negative mutant (*ΔphoA*). In *E. coli* KS272 harboring pTrcS1PhoA, alkaline phosphatase was also secreted to periplasmic space and the content of secreted alkaline phosphatase was 15.2% of total proteins.

However, most of alkaline phosphatase overexpressed under the *trc* promoter caused formation of inclusion bodies in the periplasmic space when cells were observed on the electron microscopy (data not shown). Alkaline phosphatase secreted into the periplasm could not be fractionated by the osmotic shock method (Fig. 2A). In an attempt to prevent the formation of inclusion bodies, cells were induced with lower IPTG concentrations (Fig. 2B, C). When cells were induced with 0.01 mM IPTG, alkaline phosphatase was secreted into periplasm as a soluble form without a decrease in expression level. More than 90% of alkaline phosphatase could be recovered by the osmotic shock method (Fig. 2C).

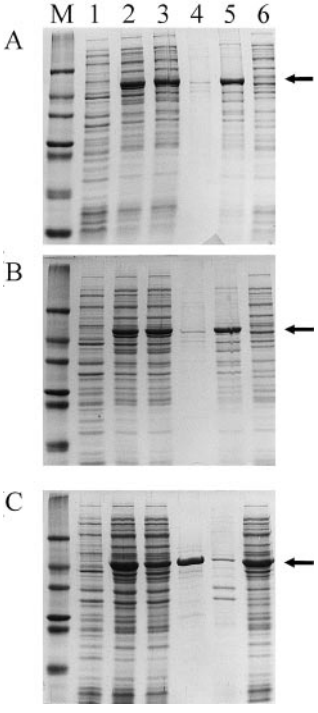

Fig. 2A–C SDS-PAGE analysis of protein samples from recombinant *E. coli* HB101 harboring pTrcS1PhoA (lanes 2–6) after inducing with **A** 1 mM, **B** 0.1 mM, **C** 0.01 mM IPTG. *Lanes: M*, molecular mass standard (bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; lactalbumin, 14.2 kDa); *1*, total proteins of host *E. coli* strain HB101 (no plasmid); *2*, total proteins; *3*, cytoplasmic proteins; *4*, periplasmic proteins; *5*, inclusion bodies; *6*, soluble proteins. *Arrows* indicate the mature form of alkaline phosphatase

The activities of alkaline phosphatase in recombinant *E. coli* HB101 and KS272 were measured after inducing with different amounts of IPTG and are summarized in Table 2. The amount of soluble alkaline phosphatase increased for both recombinant strains as the amount of inducer decreased. A considerable reduction in the formation of inclusion bodies by induction with less IPTG could be clearly seen on the SDS-PAGE (Fig. 2). The activity of alkaline phosphatase in *E. coli* HB101 (pTrcS1PhoA) was 1.38 ($\pm$0.10 SD) $\times 10^4$ units when induced with 0.01 mM IPTG, 4.45 times higher than that obtained by inducing with 1 mM IPTG. The activity of alkaline phosphatase in KS272 (pTrcS1PhoA) was considerably lower than that in HB101 (pTrcS1PhoA). The N-terminal amino acid sequence of alkaline phosphatase was determined. The five N-terminal amino acid sequence was Ala-Arg-Thr-Phe-Glu, which was consistent with the N-terminal amino acid sequence of mature alkaline phosphatase. These results indicate that the endoxylanase signal sequence was precisely processed.

Fed-batch cultivation

The pH-stat fed-batch cultures of *E. coli* HB101 and KS272 harboring pTrcS1PhoA were carried out as described in the materials and methods section. Figure 3 shows the time profiles of cell dry weight (CDW, g/l) and concentration of alkaline phosphatase (g/l) during the fed-batch cultures. To examine the effect of induction time on the production of soluble alkaline phosphatase, HB101 (pTrcS1PhoA) was induced with 1 mM IPTG at an OD_{600} of 50 or 150. When cells were induced at low cell density ($OD_{600} = 50$), the alkaline phosphatase was secreted into the periplasm and its content reached about 24.7% of total proteins at 7 h after induction. However, the alkaline phosphatase secreted formed the inclusion bodies in the periplasm (data not shown).

Table 2 Alkaline phosphatase activities in recombinant *E. coli* harboring pTrcS1PhoA induced with varying amounts of IPTG[a]

	IPTG (mM)	Activity of alkaline phosphatase (unit)[c]
E. coli HB101[b]	–	2.53($\pm$0.22)
E. coli HB101 (pTrcS1PhoA)	1	3.09($\pm$0.17) $\times 10^3$
	0.1	4.24($\pm$0.21) $\times 10^3$
	0.01	1.38($\pm$0.10) $\times 10^4$
E. coli KS272[b]	–	0
E. coli KS272 (pTrcS1PhoA)	1	1.62($\pm$0.16) $\times 10^3$
	0.1	2.06($\pm$0.11) $\times 10^3$
	0.01	2.52($\pm$0.14) $\times 10^3$

[a] All experiments were carried out in triplicate
[b] Control host cells without plasmid
[c] Unit is defined as:

$$1{,}000 \times \frac{OD_{420} - 1.75 \times OD_{550}}{\text{time} \times OD_{600}(\text{of culture})} \times \text{dilution factor}$$

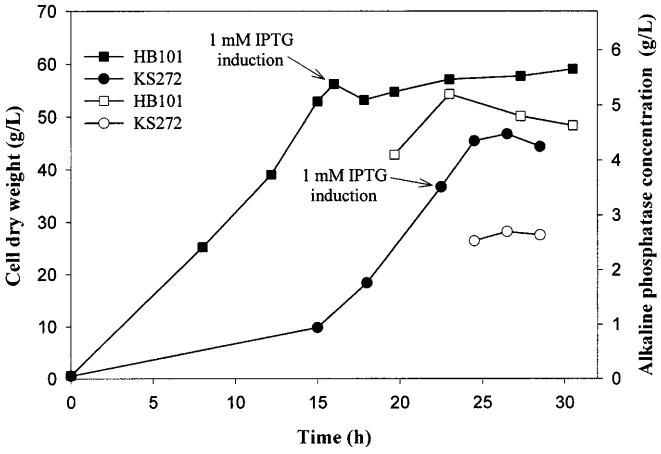

Fig. 3 Time profiles of cell dry weight and alkaline phosphatase concentrations during the fed-batch cultivation of recombinant HB101 (■,□) and KS272 (●,○) harboring pTrcS1PhoA. *Closed symbols* indicate the cell dry weight, *open symbols* indicate alkaline phosphatase concentration. *Arrows* indicate the time of induction

When cells were induced at high cell density ($OD_{600} = 150$), the cell dry weight and the content of alkaline phosphatase were 57.0 g/l and 20.3% of total proteins, respectively, at 7 h after induction (Figs. 3, 4). Most of the alkaline phosphatase secreted into the periplasm (91% of total alkaline phosphatase secreted) was in a soluble form. The concentration of soluble alkaline phosphatase was as high as 5.2 g/l at this point.

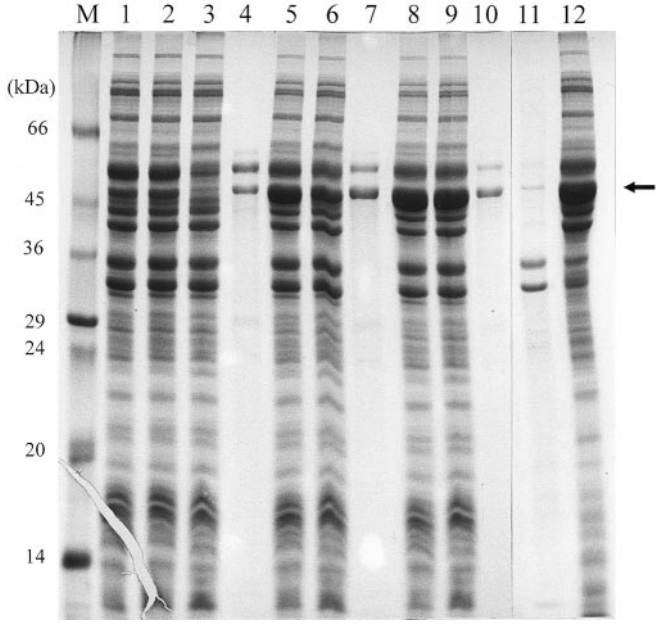

Fig. 4 SDS-PAGE analysis of protein samples from recombinant *E. coli* HB101 harboring pTrcS1PhoA. *Lanes: M*, molecular mass standard; *1–4*, proteins prepared before induction; *5–7*, proteins prepared at 4 h after induction; *8–12*, proteins prepared at 7 h after induction; *1*, *2*, *5*, *and 8*, total proteins; *3*, *6*, *and 9*, cytoplasmic proteins; *4*, *7*, and *10*, periplasmic proteins; *11*, inclusion bodies; *12*, soluble proteins. The *arrow* indicates the mature alkaline phosphatase

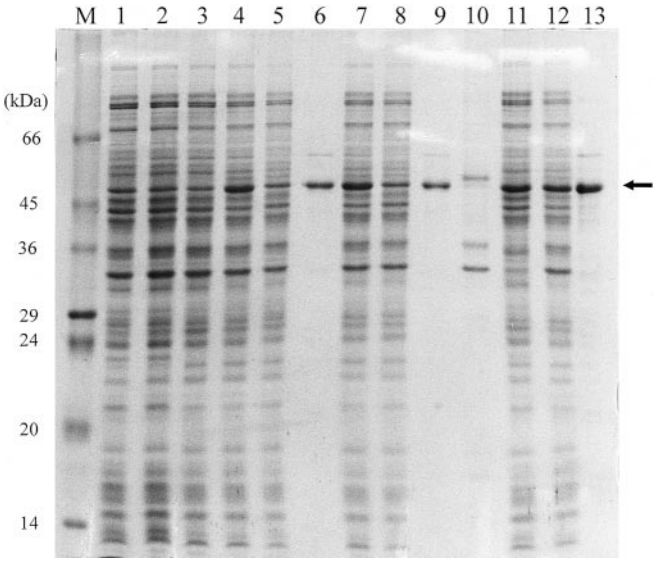

Fig. 5 SDS-PAGE analysis of protein samples from recombinant *E. coli* KS272 harboring pTrcS1PhoA. *Lanes: M*, molecular mass standard; *1–3*, proteins prepared before induction; *4–6*, proteins prepared at 2 h after induction; *7–11*, protein prepared at 4 h after induction; *12 and 13*, proteins prepared at 6 h after induction; *1*, *2*, *3*, *4*, *7*, and *12*, total proteins; *5* and *8*, cytoplasmic proteins; *6*, *9*, and *13*, periplasmic proteins; *10*, inclusion bodies; *11*, soluble proteins. The *arrow* indicates the mature alkaline phosphatase

E. coli KS272 harboring pTrcS1PhoA was induced with 1 mM IPTG at an OD_{600} of 100 (Fig. 3). The cell dry weight and the content of alkaline phosphatase were 46.7 g/l and 15.9% of total proteins, respectively, at 4 h after induction (Figs. 3, 5). Alkaline phosphatase was produced in a soluble form, and 83% of alkaline phosphatase secreted (2.7 g/l) could be recovered from the periplasm by the osmotic shock method.

Discussion

Secretory production of recombinant proteins has several advantages. For the secretion of recombinant proteins in *E. coli*, various signal sequences have been examined (Shibui et al. 1991; Klein et al. 1992; Barthelemy et al. 1993; Lucic et al. 1998). However, the presence of a signal peptide has not always ensured the efficient secretion of recombinant proteins. In this study, we used a new signal sequence cloned from *Bacillus* sp. endoxylanase gene (Jeong et al. 1998). Using this endoxylanase signal sequence, the *Bacillus* sp. endoxylanase and *E. coli* alkaline phosphatase could be secreted (up to 25.3%) into the periplasm of *E. coli* using the inducible *trc* promoter. Furthermore, our recent study showed that human leptin could also be effectively secreted into the periplasm of *E. coli* using this endoxylanase signal sequence (data not shown). These results suggest that the *Bacillus* sp. endoxylanase signal sequence can be used for the secretory production of recombinant proteins in various *E. coli* strains.

Large-scale production of recombinant proteins is generally achieved by fed-batch cultivation. Many recombinant proteins can be produced in the form of inactive inclusion bodies as well as in an active soluble form. Production of proteins in a soluble form is beneficial because protein purification is easier than purification from inactive inclusion bodies. Production of recombinant proteins by high-cell-density cultivation can be affected by several factors such as culture temperature and IPTG concentration (Wong et al. 1998). It is well documented that production of soluble proteins is promoted by culturing at reduced temperature (Makrides 1996). However, cultivation of HB101 (pTrcS1PhoA) at 30 °C did not increase soluble alkaline phosphatase fractionation significantly (data not shown). Therefore, fed-batch culture of HB101 (pTrcS1PhoA) was carried out at 37 °C. KS272 (pTrcS1PhoA) was cultivated at 30 °C because it cannot be grown to high cell density at high temperature (Park et al. 1999). Both recombinant strains were induced with 1 mM IPTG, which was found to be appropriate in high-cell-density culture of recombinant *E. coli*. It is also well known that the time point of induction significantly affects protein production in high-cell-density cultivation (Lee 1996). We also wanted to examine whether the time point of induction affects the amount of soluble protein produced by secretion. For recombinant *E. coli* HB101 harboring pTrcS1PhoA, IPTG induction at low cell density ($OD_{600} = 50$) resulted in the formation of inclusion bodies although the content of secreted alkaline phosphatase (24.7% of total proteins) was slightly higher than that (20.3%) obtained by induction at high cell density ($OD_{600} = 150$). However, IPTG induction at high cell density ($OD_{600} = 150$) resulted in the formation of soluble proteins and a high efficiency of fractionation (Fig. 4). Although the reason for this is not clear, it may be due to the lower IPTG dosage per cell observed in flask cultures.

In this report, we described the use of *Bacillus* sp. endoxylanase signal peptide for the secretory production of *Bacillus* sp. endoxylanase and *E. coli* alkaline phosphatase in *E. coli*. We also demonstrated that alkaline phosphatase could be efficiently secreted in high-cell-density fed-batch fermentation. This is important because few studies have described secretory protein production at high cell densities. Therefore, the *Bacillus* sp. endoxylanase signal peptide seems to be useful for the high-level secretory production of various recombinant proteins in *E. coli*.

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