Secretory Production of Erythropoietin and the Extracellular Domain of the Erythropoietin Receptor by *Bacillus brevis*: Affinity Purification and Characterization

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*Bacillus brevis* secretes a large amount of cell wall proteins into the culture medium. For construction of *Bacillus brevis* expression-secretion vectors of human erythropoietin (EPO) and the extracellular domain of mouse erythropoietin receptor (sEPOR), cDNA for each mature form was inserted into a plasmid containing the promoter region and the signal-peptide encoding region of a cell wall protein. Culture supernatants of transformants were affinity purified using a monoclonal antibody-fixed gel for EPO and an EPO-fixed gel for sEPOR. The affinity purification efficiently removed unwanted proteins, giving samples with sufficiently high purity to analyze amino acid sequences of N-terminal regions and biological activities. Combination of this secretory production and affinity purification may facilitate isolation of a large amount of pure EPO and sEPOR, and is useful for further understanding the molecular mechanism of interaction between EPO and EPOR.

Key words: erythropoietin; soluble erythropoietin receptor; *Bacillus brevis*; secretory production; affinity purification

EPO plays a key role in regulation of red blood cell production by stimulating differentiation and growth of erythroid precursor cells, and supporting their survival.\(^1\)  EPO is produced by the kidney and the erythropoietin gene is located on the short arm of chromosome 7 in humans.\(^2\)  The kidney is the major production site of EPO in adults, and the kidney EPO travels through the blood circulation to act on target cells in the erythropoietic tissues such as bone marrow and spleen. In addition to the function in erythropoiesis, EPO may function in the CNS as a neurotrophic factor.\(^3\)\(^4\)  The blood-brain barrier insulates the CNS from the circulation and therefore blood EPO produced by the kidney does not interact with neurons in the CNS. Since EPO is produced by astrocytes and EPOR is expressed in neurons, EPO in the CNS acts on neurons in a paracrine fashion.\(^5\)\(^6\)\(^7\)

EPO is a heavily glycosylated protein; N-glycosylation of human EPO occurs at positions 24, 38, and 83 of a total of 165 amino acid residues, and O-glycosylation does at position 126.\(^8\)\(^9\)\(^10\)  All of the N-linked carbohydrates are complex-type. Studies of biological functions of the carbohydrates in EPO using enzymatically deglycosylated EPOs or glycosylation-defective mutant EPOs, showed that the sialylated N-linked carbohydrates are necessary for the *in vivo* activity but glycosylation is not required for the *in vitro* activity.\(^11\)\(^12\)\(^13\)\(^14\)  N-Linked carbohydrates with terminal sialic acids confer stability on EPO not to be eliminated from the circulation. EPOR consists of an extracellular 225–226 amino acid domain, a 22 amino acid transmembrane region, and an intracellular 236 amino acid domain.\(^15\)\(^16\)\(^17\)\(^18\)  A single N-glycosylation site is present in the extracellular domain in EPOR. N-Glycosylation of EPOR is not required for binding with EPO nor for stimulating proliferation of EPO-dependent cells.\(^19\)\(^20\)  An extracellular domain of EPOR, sEPOR, has been shown to bind with EPO and the binding does not require N-glycosylation of sEPOR.\(^21\)\(^22\)\(^23\)\(^24\)\(^25\)\(^26\)\(^27\)\(^28\) Thus, glycosylation is not needed in either EPO or sEPOR for their interaction, indicating that the interaction between EPO and sEPOR can be investigated using the recombinant products of procaryotes. Development of the system for producing these non-glycosylated forms would be useful for analysis of the ligand-receptor complex with X-ray crystallography, because difficulties due to sugar-chain microheterogeneity can be eliminated.

*B. brevis* 47 secretes a large amount of cell wall proteins (OEP and MWP) into the culture medium.\(^29\)\(^30\)  The genes for these proteins constitute a single operon called cwp. An efficient production of heterologous proteins by *B. brevis* has been shown using the promoter region of cwp operon and the signal-peptide encoding region of the MWP gene for construction of expression-secretion vectors.\(^31\)\(^32\)\(^33\)\(^34\)\(^35\)\(^36\) This system may provide much useful information on the structure-function relationships in the EPO-EPOR interaction by complementing production systems described previously. In this paper, we describe isolation of EPO and sEPOR produced by *B. brevis* and their basic properties, mainly focusing on the feasibility of this system with little attempts to optimize conditions for efficient production.

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Abbreviations: EPO, erythropoietin; CNS, central nervous system; EPOR, erythropoietin receptor; sEPOR, soluble erythropoietin receptor; OWP, outer wall protein; MWP, middle wall protein; APMSF, (p-amidinephosphophenyl) methanesulfonil fluoride; BSA, bovine serum albumin; PBS, 10 mM phosphate buffered saline; pH 7.4; BHK, baby hamster kidney; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.
Materials and Methods

Construction of plasmids and cell culture. Complementary DNAs of human EPO\textsuperscript{33} and murine sEPO\textsuperscript{29} were prepared as described previously. Nucleotide sequences that correspond to N-terminal sequence of human EPO cDNA or mouse sEPO cDNA were mutated to create Bsp126I site so that the DNA fragment coding the mature peptide of EPO or sEPO could be ligated to PatI site at the end of MWP signal peptide coding sequence without changing the amino acid sequences of EPO or sEPO mature peptide when the MWP signal peptide was cleaved at the appropriate site after translation in B. brevis (Fig. 1). Finally, the DNA fragment covering from this newly created Bsp126I site to the BglII site (in the 3’ noncoding region), which included the coding region of mature EPO peptide, was inserted into the PstI-BglII site of expression vector pNU210EO\textsuperscript{16} yielding pNU210EO\textsuperscript{16}. Likewise, the DNA fragment covering from the Bsp126I site to the XhoI site located just after the stop codon in pmEPRsolDHFR\textsuperscript{27} which included the coding region of mature sEPO, was inserted into the PstI-XhoI site, creating pNU210sEPO. Bacillus brevis strain HPD31\textsuperscript{17} was transformed with pNU210EPO or pNU210sEPO with the Tris-polyethylene glycol method.\textsuperscript{26} For production of EPO or sEPO, transformants were grown in 4 ml of No. 9 medium, pH 7.0, consisting of 4% polyethylene, 0.5% meat extract, 0.2% yeast extract, 1% glucose, and 0.01% uracil, at 30°C for 2 days and culture supernatants were recovered.

Purification of EPO and sEPO produced by B. brevis. To stabilize EPO or sEPO in culture supernatant, 0.01% Tween 20, 10 μm leupeptin, 10 μM APMF, and 0.1% BSA were added to supernatants. EPO was purified from 600 ml of culture supernatant using an anti-EPO monoclonal antibody R2-fixed Sepharose column.\textsuperscript{29} EPO absorbed to the column was eluted with 0.2 M acetic acid, pH 2.5, containing 0.4 M NaCl, 0.01% Tween 20, 10 μm leupeptin, and 10 μM APMF. The eluted EPO preparation was neutralized with 3 M Tris, sEPO was purified from culture supernatant using an EPO-fixed gel.\textsuperscript{25} sEPO absorbed to the gel was eluted with PBS containing 1.5 M MgCl\textsubscript{2} and 0.01% Tween 20. Purified EPO and sEPO were dialyzed against PBS containing 0.01% Tween 20, and used for assay of their biological activity.

EPO and sEPO produced by mammalian cells. Glycosylated forms of recombinant human EPO and mouse sEPO were produced by BHK cells\textsuperscript{25} and CHO cells,\textsuperscript{25} respectively, and they were isolated as described previously.\textsuperscript{22,23} EPO fully deglycosylated by glycosidases,\textsuperscript{23} mutant EPO that are defective in all of three N-glycosylation sites,\textsuperscript{19} EPO produced by tobacco cells,\textsuperscript{38} and human urinary EPO\textsuperscript{39,40} were prepared as described previously.

Assay of EPO and sEPO proteins, and their biological activity. EPO protein was measured by EIA in which two monoclonal antibodies recognizing different antigenic determinants of EPO were used.\textsuperscript{33} sEPO protein was measured by EIA using monoclonal antibody and polyclonal antibody.\textsuperscript{10} Biological activity of EPO was assayed using the erythroleukemia cell line EP-FDC-P2 that requires EPO for growth.\textsuperscript{41} The cell growth was measured colorimetrically by MTT cleavage.\textsuperscript{41} Binding of sEPO with EPO was assayed by estimating the inhibitory effect of sEPO on EPO-induced cell growth EP-FDC-P2.

Other methods. Western blotting analysis of sEPO was done as described previously\textsuperscript{26} using the rabbit antisera against sEPO produced by CHO cells. The amino acid sequences of N-terminal regions of EPO and sEPO were measured with an ABI 477A automatic sequencer (Applied Biosystems, U.S.A.), using about 1 μg of protein.

Results and Discussion

Isolation and characterization of EPO produced by B. brevis

EPO in culture media of B. brevis was purified using the gel to which EPO-directed monoclonal antibody was fixed. EPO absorbed to the gel was eluted under acidic conditions (pH 2.5).\textsuperscript{37} The culture medium contained 2 μg/ml of EPO, which was purified with 300-fold purification and 30% activity recovery. EPO proteins purified from various sources including B. brevis were analyzed with SDS-polyacrylamide gel electrophoresis; proteins were made visible with silver staining (Fig. 2). The glycosylated forms of human urinary EPO and recombinant human EPO produced by BHK cells (BHK-EPO) migrated with a size of 35 kDa, while BHK-EPO fully deglycosylated by glycosidases\textsuperscript{17} migrated with a size of 18 kDa. A mutant BHK-EPO in which all of three N-glycosylation sites were defective\textsuperscript{17} had a size of 19 kDa, which was the O-glycosylated form. Tobacco cells produced a glycosylated EPO with a size of 31 kDa but it was not sialylated.\textsuperscript{38} The gel to which EPO-directed monoclonal antibody was fixed was quite efficient for purification of EPO produced by B. brevis; this one-step purification provided nearly homogeneous EPO. EPO produced by B. brevis migrated with a size of 18 kDa, a similar size to that of the fully deglycosylated BHK-EPO.

We sequenced the N-terminal region of B. brevis-EPO and found the sequence APPRLI, which was identical with 1 2 3 4 5 6

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig1.png}
\caption{Design of Expression Vectors for Production of Human EPO and Mouse sEPO by B. brevis. (A) indicates sequences of nucleotides and amino acids of the region where MWP signal sequence and mature EPO coding sequence were connected. PatI site located at the cleavage site of MWP signal-peptide coding sequence in pNU210 was used for ligation of the mature EPO coding region. (B) indicates sequences of nucleotides and amino acids of the region where MWP signal sequence and mature sEPO coding sequence were connected. The PatI site in pNU210 was also used for ligation of the mature sEPO coding region. Dotted lines indicate cleavage sites between MWP signal peptide and mature proteins. Marked nucleotides show the sugar moieties that have been modified.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{SDS-Polyacrylamide Gel Electrophoresis of Human EPO. Lane 1, human urinary EPO\textsuperscript{29,40}; lane 2, EPO produced by BHK cells,\textsuperscript{25} lane 3, fully deglycosylated BHK-EPO\textsuperscript{17}; lane 4, mutant BHK-EPO that is defective for all of three N-glycosylation sites,\textsuperscript{17} lane 5, EPO produced by tobacco cells,\textsuperscript{26} lane 6, EPO produced by B. brevis. Approximately 1 μg of EPO was put on, separated in 12% gel, and stained with silver.}
\end{figure}
the sequence of mature human EPO, indicating that the MWP signal peptide was cleaved off at the desired position.

Biological activity of B. brevis-EPO was compared with that of BHK-EPO using the EP-FDC-P2 cells that require EPO for their growth. Cell growth was estimated by measurement of MTT cleavage, an index of cell survival and proliferation. As Fig. 3 shows, B. brevis-EPO supported proliferation of the cells with a similar potency to that of BHK-EPO. The biological activity of both EPO was completely abrogated by addition of an excess of sEPOR produced by CHO cells.

**Isolation and characterization of sEPOR produced by B. brevis**

sEPOR in culture media of B. brevis was purified using the gel to which BHK-EPO was fixed. sEPOR absorbed to the gel was eluted by 1.5 M MgCl₂. The culture medium contained 5 μg/ml of sEPOR, which was purified with 200-fold purification and 20% protein recovery. Figure 4A shows staining of the eluted proteins with Coomassie blue after separation by SDS–polyacrylamide gel electrophoresis. A major sEPOR produced by CHO cells (CHO-sEPOR) migrated with a size of 33 kDa but there was a faint band of 29 kDa. sEPOR has one N-glycosylation site; the 33 kDa protein is a glycosylated sEPOR and the 29 kDa protein is an unglycosylated sEPOR. Affinity purification of B. brevis-sEPOR with EPO-fixed gel successfully removed unwanted proteins but this purification yielded two clear bands with sizes of 29 kDa and 28 kDa. The culture medium of parental B. brevis containing no plasmid was affinity purified and the fraction eluted by MgCl₂ was analyzed with SDS–polyacrylamide gel electrophoresis. There was no band with 29 kDa nor 28 kDa (data not shown). When the purified B. brevis-sEPOR preparation were analyzed with Western blotting using antibody against CHO-sEPOR, both components reacted with the antibody (Fig. 4B), suggesting that both proteins are sEPOR. This was confirmed by analysis of the amino acid sequence of both proteins as described below.

The putative two sEPOR proteins in the purified B. brevis-sEPOR preparation were separated with SDS–polyacrylamide gel electrophoresis and the gel was sliced into 1-mm lengths. Proteins were extracted electrophoretically from the sliced gels and the extracted proteins were sequenced. Both proteins gave the identical N-terminal sequence, APSPSLPDP, which agreed with the sequence of the mature mouse sEPOR. From their molecular size, the 29-kDa sEPOR probably is the full length of sEPOR because this size is consistent with that of unglycosylated sEPOR produced by CHO cells. The 28-kDa sEPOR might result from proteolytic digestion of the 29-kDa sEPOR but the amino acid sequences of C-terminal region of both proteins remain to be examined.

Binding of B. brevis-sEPOR to EPO was assayed using its inhibitory effect on EPO-induced cell growth and com-

![Graph](image)

Fig. 3. Biological Activity of EPO Produced by BHK Cells and B. brevis.
Activity of EPO produced by BHK cells (●, ○) and EPO produced by B. brevis (■, □); activity assayed in the absence (●, ■) or presence (○, □) of sEPOR produced by CHO cells. When assayed in the presence of sEPOR, 3000-fold excess of sEPOR was added simultaneously with EPO. Cleavage of MTT was estimated by measurement of absorbance at 600 nm using the cells that their growth requires EPO as described in Materials and Methods.

![Diagram](image)

Fig. 4. SDS–Polyacrylamide Gel Electrophoresis and Western Blotting of sEPOR.
Twelve % gel was used for protein separation. In (A), gel was stained with Coomassie blue. Lane 1, sEPOR (2 μg) produced by CHO cells; lane 2, sEPOR (2 μg) produced by B. brevis. In (B), sEPOR was detected with Western blotting using the antisera against CHO-sEPOR. Lane 1, CHO-sEPOR (40 ng); lane 2, CHO-sEPOR (20 ng); lane 3, B. brevis-sEPOR (30 ng); lane 4, B. brevis-sEPOR (40 ng).
pared with that of CHO-sEPOR. As Fig. 5 shows, B. brevis-sEPOR inhibited EPO-dependent cell growth but its potency was somewhat lower than that of CHO-sEPOR. It is not known if this low inhibitory effect is due to the presence of the 280kDa sEPOR, which might have a lower affinity for EPO than does the full-length sEPOR.

Production of EPO by 227 and sEPOR by 227 by procaryotic cells has been reported using E. coli, by which they were produced as intracellular proteins. The recombinant products were largely insoluble and a small soluble fraction was used for further analyses including purification. Further refolding of the protein solubilized under denaturing conditions was required. Further, they were produced as fusion proteins, fused with glutathione-S-transferase or with oligo-His for affinity purification, and removal of these tags is necessary for further analyses. B. brevis secreted EPO and sEPOR into the culture medium after removal of the signal sequences at the desired positions. One-step purification of EPO with monoclonal antibody-fixed gel, and sEPOR with EPO-fixed gel efficiently yielded the proteins with high purity sufficient for analyses of their basic properties. Secretory production and affinity purification of EPO and sEPOR will be useful to analyze the structure-function relationships in the EPO-EPOR interaction. The culture conditions for high production require further study.

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References