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# Signal peptide design for improving recombinant protein secretion in the baculovirus expression vector system

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ABSTRACT

Almost all secretory proteins have a sequence consisting of 15–30 amino acids at the N-terminus (the so-called N-terminal signal peptide). Signal peptides direct the propeptide to the endoplasmic reticulum and through the secretory pathway. Although the sequences of signal peptides vary greatly, all contain a basic amino acid in the N-terminal region, followed by a hydrophobic core region. With the aim of improving the level of secretion of recombinant proteins in the baculovirus expression vector system (BEVS), we designed several signal peptides based on the signal peptide of silkworm SP1 by introducing the basic amino acid arginine into the N-terminal region and/or the polar amino acid asparagine into the C-terminal region of the silkworm SP1 signal peptide. Human interleukin (IL)-4, IL-13, and the extracellular domain of human IL-11 receptor α1 (IL-11Rα1) were fused to wild-type and modified SP1 signal peptides, and the effects that each signal peptide had on secretion were measured by enzyme-linked immunosorbent assay. Introduction of the basic amino acid arginine into the N-terminal region did not result in an increase in secretion of the recombinant proteins. On the other hand, introduction of the polar amino acid asparagine into the C-terminal region enhanced secretion of the recombinant proteins. Therefore, it is suggested that polar amino acids in the C-terminal region of signal peptides are important in the secretion of recombinant proteins in BEVS.



## Introduction

The baculovirus expression vector system (BEVS) is a convenient system that provides for producing large quantities of recombinant proteins. BEVS provides high expression levels, disulfide bond formation, processing of recombinant proteins, oligomerization, and post-translational modifications [1–4]. However, when the target proteins in BEVS are membrane or secretory proteins, they are sometimes insoluble and poorly processed.

The N-terminus of newly synthesized secretory proteins contains a sequence consisting of 15–30 amino acids referred to as the signal peptide. Signal peptides direct the propeptide to the endoplasmic reticulum and through the secretory pathway [5–7]. Signal peptides all contain a basic amino acid in the N-terminal region and a hydrophobic core region [8]. It has been proposed that the positively charged basic amino acid in the N-terminal region is involved in the interaction with the negatively charged membrane of the endoplasmic reticulum [9], and a recent report showed that, in yeast, introduction of basic amino acids into the N-terminal region of the sequence enhances the secretion of recombinant proteins [10–12]. These studies indicate that positively charged basic amino acids in the N-terminal region play an important role in the translocation of newly synthesized proteins. On the other hand, several abundantly secreted silkworm proteins have certain other characteristics in common: as well as basic amino acids in the N-terminal region they all have polar amino acids in the C-terminal region. Therefore, it is suggested that polar amino acids in the C-terminal region also play an important role in the translocation mechanisms. It is hoped that by clarifying the structural requirements of the signal peptides in BEVS a more efficient system for the expression of secretory proteins can be established. Accordingly, we designed a number of signal peptides with the hope of increasing the secretion yield. Here we elucidate the importance of the basic amino acids in the N-terminal region and the polar amino acids in the C-terminal region.

## Materials and methods

*Cloning and expression of recombinant proteins.* The genes of human IL-4 (hIL-4) [13–16], human IL-13 (hIL-13) [17–20], and the extracellular domain (ECD) of human IL-11 receptor (hIL-11Rα1) [21–23] were amplified for insertion into the expression

*Abbreviations:* BEVS, baculovirus expression vector system; ECD, extracellular domain; IL, interleukin; MOI, multiplicity of infection

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vector as described elsewhere (Futatsumori-Sugai et al., manuscript in preparation).

The entire coding regions, including the native signal peptides, of hIL-4 and hIL-13 were independently subcloned into a pM23 transfer vector (Katakura Industries, Inc.) to attach a FLAG tag at the C-terminus. Each signal peptide DNA sequence was fused into a pM01 transfer vector (Katakura Industries, Inc.), designated sig-pM01. The regions of hIL-4 (residues 25–153) and hIL-13 (residues 35–146) not including the native signal peptide were independently cloned into each sig-pM01 vector.

The DNA sequence of the human IgG Fc region was fused into a pM01 transfer vector (designated pM01-Fc). The extracellular domain gene of human IL-11Rα1 (residues 1–363), designated hIL-11Rα1(ECD), including the native signal peptide, was cloned into pM01-Fc to fuse the Fc domain to the C-terminus. The ECD region of IL-11Rα1 (residues 23–363) not including the native signal peptide and each of the engineered signal peptide sequences were inserted into pM01-Fc vectors.

*Cell culture, recombinant baculovirus production, and expression.* Silkworm BmN cells [24] were maintained at 25 °C in flasks containing TC-100 culture medium (Sigma) supplemented with 10% fetal calf serum (FCS). Cells were co-transfected with linearized baculovirus (BmNPV strain CPd) [25] DNA (200 ng) and transfer vector DNA (500 ng) by using Lipofectin (Invitrogen) and were incubated at 25 °C. The selection of each single recombinant baculovirus and tittering were performed by limiting dilution. For expression, BmN cells were seeded into 6-well plates ($1 \times 10^6$ cells/well) in FCS-free medium. The cells were infected with recombinant viruses at a multiplicity of infection (MOI) of 7.5 at 25 °C for 72 h. Following the infection, BmN cells were removed by centrifugation at 3000*g* at 4 °C for 10 min.

*ELISA.* The yields of hIL-4/FLAG, hIL-13/FLAG, and hIL-11Rα1(ECD)/Fc in the culture medium were measured by ELISA. Flat-bottomed 96-well plates were first coated with 100 μL of medium diluted with phosphate buffered saline (PBS) at room temperature for 1 h. Each well was blocked with 300 μL of PBS-T containing 1% (w/v) BSA for 1 h at room temperature.

To detect FLAG-fusion proteins, polyclonal anti-FLAG antibody (Sigma) diluted 1:10,000 in PBS-T containing 1% BSA was added, the plates were incubated for 1 h at room temperature, and the plates were then washed three times with PBS-T. HRP-conjugated goat anti-rabbit IgG (Beckman Coulter) diluted 1:5000 in PBS-T containing 1% BSA was added and the plates were incubated at room temperature for 1 h. The plates were washed three times with PBS-T, and 100 μL/well of TMB was added. The reactions were terminated by adding phosphoric acid, and the absorbance at 450 nm was measured by use of a microplate reader (Bio-Rad Laboratories).

To detect Fc-fusion proteins, HRP-conjugated goat anti-human IgG (Beckman Coulter) diluted 1:5000 in PBS-T containing 1% BSA was added, and the plates were incubated at room temperature for 1 h. The plates were washed three times with PBS-T, and 100 μL/well of TMB was added. The reactions were terminated by adding phosphoric acid, and the absorbance at 450 nm was measured by use of a microplate reader.

*Western blot analysis.* Western blot analysis was performed to measure the total expression levels of hIL-4/FLAG, hIL-13/FLAG, or hIL-11Rα1(ECD)/Fc in silkworm BmN cells. Infected BmN cells were solubilized in SDS–PAGE sample buffer, and aliquots of the total cell lysates were subjected to Western blot analysis using anti-FLAG antibody or anti-human IgG antibody. Detection was with ECL reagents (GE healthcare bioscience).

*Binding assay.* The binding assay of FLAG tagged hIL-4 and hIL-4R/Fc, or FLAG tagged hIL-13 and hIL-13R/Fc was performed by sandwich ELISA. FLAG tagged hIL-4 (SP1–2 signal) and FLAG tagged hIL-13 (SP1–2 signal) were expressed in silkworm BmN cells and purified by anti-FLAG affinity. Native proteins, hIL-4 (native) and hIL-13 (native), were purchased from R&D. The specific receptors fused with Fc fragment of human IgG1, hIL-4R/Fc and hIL-13R/Fc (Tsumoto, unpublished results), were expressed in silkworm larvae, and purified by HiTrap rProtein A FF (GE healthcare bioscience). Flat bottom 96-well plates were coated with hIL-4R at 500 ng/well or hIL-13R at 1 μg/well diluted with PBS at room temperature for 1 h. Each well was blocked with 300 μL of PBS-T containing 1% (w/v) BSA overnight at 4 °C. Recombinant hIL-4 or hIL-13 in PBS-T containing 0.1% BSA was added, incubated for 1hr at room temperature, and washed with PBS-T three times. Afterwards polyclonal anti-FLAG antibody (sigma) or biotinylated anti IL-13 antibody (R&D) diluted 1:10,000 in PBS-T containing 1% BSA was added, incubated for 1hr at room temperature, and washed with PBS-T 3 times. HRP-conjugated goat anti-rabbit IgG (Beckman Coulter) or HRP-conjugated streptavidine (Beckman Coulter) diluted 1:15,000 in PBS-T containing 0.1% BSA was added and plates were incubated at room temperature for 1hr. The plates were washed PBS-T three times and 100 μL/well of TMB was added. The reactions were terminated by adding phosphoric acid and the absorbance was measured by microplate reader (Bio-Rad Laboratories) at 450 nm.

## Results and discussion

### *Measurement of relative secretion levels in BEVS*

The signal peptides of several abundantly secreted silkworm proteins have certain features in common: in addition to the basic amino acids in the N-terminal region and hydrophobic amino acids in the core region, they contain polar amino acids in the C-terminal region (Table 1).

In this study, we used the signal peptide from SP1 because SP1 is abundantly secreted into silkworm hemolymph. We introduced the basic amino acid arginine into the N-terminal region and/or the polar amino acid asparagine into the C-terminal region of the silkworm SP1 signal peptide (Table 2), and we studied the influences that each signal peptide had on secretory expression in BEVS. The human proteins fused with each signal peptide were expressed in the silkworm cells by means of a baculovirus, and the relative secretion levels of the human proteins into the culture medium were measured by ELISA.

First, with regard to wild-type signal peptides, the signal peptides of SP1 was more effective than the native signal peptides in terms of hIL-4 and hIL-11Rα1(ECD) secretion (Fig. 1, columns 30K and SP1), suggesting that the SP1 signal peptide is effective for the secretory expression of many recombinant proteins. The native signal peptide of hIL-13 resulted in the best yield of hIL-13 secretions in silkworm cells (Fig. 1B). The structure of the native signal peptide of hIL-13 is similar to the structures of insect signal peptides (the basic amino acid is in the N-terminal region, and polar amino acids are localized in the C-terminal region; Table 2), and

**Table 1**
Signal peptides of major silkworm secretory proteins.

| Protein name | Accession number | Signal peptide sequence |
|---|---|---|
| 30K | X07552 | MRLTLFAFVLAVCALASNA |
| SP1 | NM_001113276 | MRVLVLLACLAAASA |
| SP2 | NM_001044125 | MKSVLILAGLVAVALSSAVPKP |
| Bombyxin A-4 | NM_001128135 | MKILLAIALMLSTVMWVST |
| Vitellogenin | AB239763 | MKLFVLAAIIAAVSS |
| Lysozyme | NM_001043983 | MQKLIIFALVVLCVGSEA |
| Chitinase precursor | AB052914 | MRAIFATLAVLASCAALVQS |

The basic amino acids are shown in green, and the polar amino acids are shown in red.

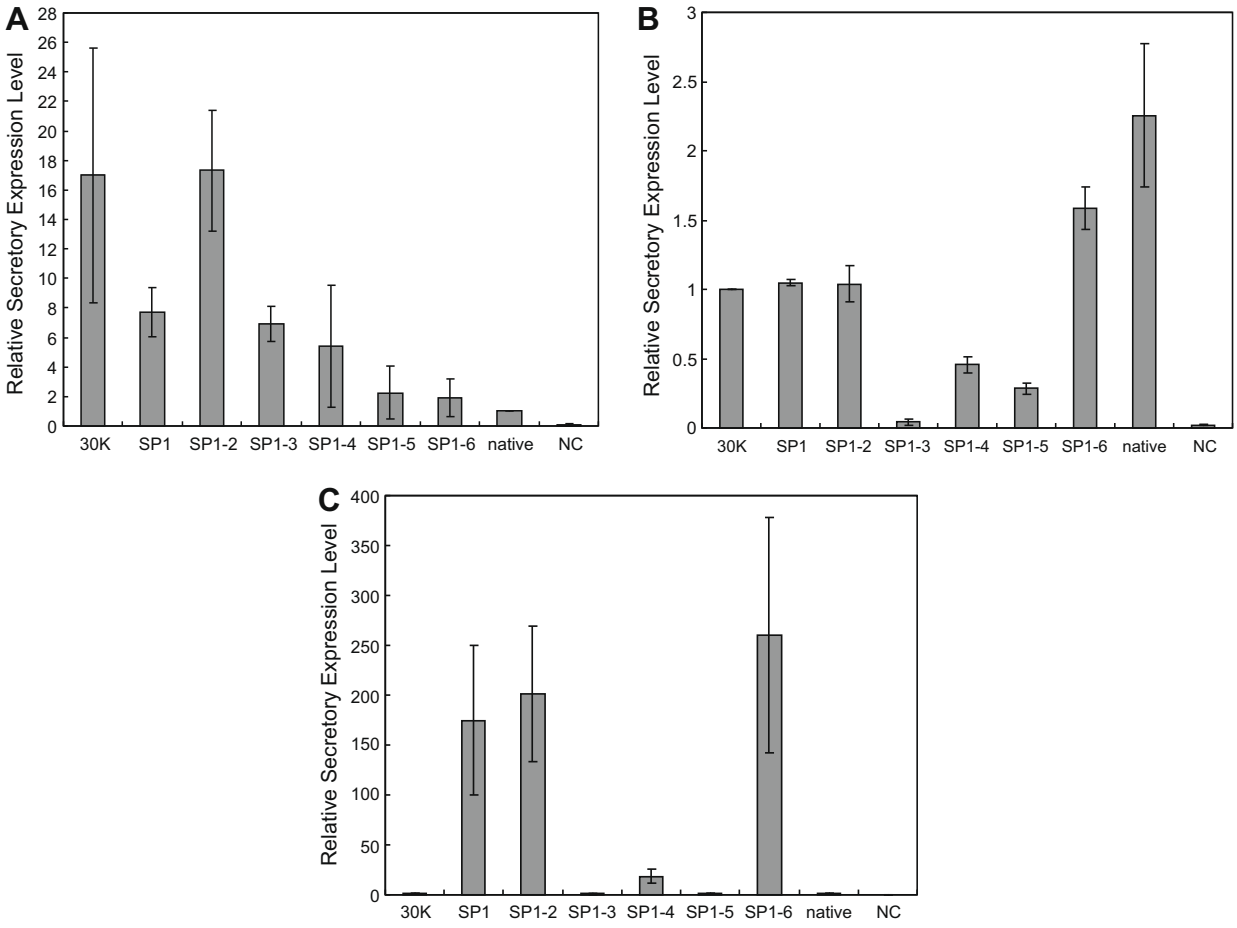


**Fig. 1.** Relative secretion levels of human IL-4 (A), human IL-13 (B), and human IL-11Rα1(ECD) (C). Relative secretion level of each signal peptide to the native one (A and C) or to 30K was measured as described in Materials and methods. Errors are standard deviations of five independent experiments. NC: negative control, which is non-infected cell medium.

**Table 2**
Signal peptide sequences used in this study.

| Name | Sequence |
|---|---|
| 30K | MRLTLFAGVLAVCALASNA |
| SP1 | MRVLVLLACLAAASA |
| SP1–2 | MRVLVLLACLAAASNA |
| SP1–3 | MRRVLVLLACLAAASA |
| SP1–4 | MRRVLVLLACLAAASNA |
| SP1–5 | MRRRVLVLLACLAAASA |
| SP1–6 | MRRRVLVLLACLAAASNA |
| IL-4 native | MGLTSQLLPPLFFLLACAGNFVHG |
| IL-13 native | MHPLLNPLLLALGLMALLLTTVIALTCLGGFASP |
| IL-11Rα1 native | MSSSCSGLSRVLVAVATALVSA |

The basic amino acids are shown in green, and the polar amino acids are shown in red.

therefore the native signal peptide of hIL-13 led to efficient secretion in the silkworm cells.

Next, with regard to the effect of modified signal peptides on the expression of hIL-4, the signal peptide in which asparagine had been introduced into the C-terminal region (SP1–2) resulted in a marked increase in the secretion level of hIL-4 in the silkworm cells compared to the secretion level with the native and SP1 signal peptides (Fig. 1(A)). However with SP1–3 and SP1–5, in which a positive charge was introduced into the N-terminal region, the secretion level of hIL-4 was lower than that with SP1. These results suggest a difference between yeast [10] and insects in the importance of a positive charge in the N-terminal region. The secretion yield was also low with the native signal peptide of hIL-4. The structure of the native signal peptide is distinctly different from the structure of the silkworm signal peptides: polar amino acids are concentrated in the N-terminal region, and a basic amino acid is localized in the C-terminal region (Table 2).

The relative secretion levels of hIL-13 into the culture medium to 30K were lower with SP1–3 and SP1–5 than with SP1 (Fig. 1(B)), as was also the case for hIL-4, whereas the secretion levels of hIL-13 with SP1–2, in which polar amino acids are introduced into the C-terminal region, were identical to that of the 30K signal peptide.

Finally, the relative secretion levels of hIL-11Rα1(ECD) into the culture medium to the native signal peptide were measured. The signal peptide of SP1–6 resulted in the highest secretion level (Fig. 1(C)). The low secretion of hIL-11Rα1(ECD) with its native signal peptide is thought to result from the difference between its structure and that of the insect signal peptides. The levels of hIL-11Rα1(ECD) secretion were lower with SP1–3 and SP1–5 than with SP1. As was also seen with hIL-4, the hIL-11Rα1(ECD) secretion level was higher with SP1–2 than with SP1–3 and the secretion level was higher with SP1–4 than with SP1–5. Thus, the secretion levels of hIL-4 and hIL-11Rα1(ECD), which declined when a basic amino acid was introduced into the N-terminal region of the signal peptide, recovered when a basic amino acid was removed from the N-terminal region and a polar amino acid was introduced into the C-terminal region, a result suggesting that these polar amino acids play a key role in silkworm secretory mechanisms.

*Measurement of total expression level in silkworm cells*

Total cell lysates from baculovirus-infected cells were subjected to Western blot analysis in order to examine the relationship between the relative levels of secretion into the culture medium and the total expression of proteins fused with each of the signal peptides (Fig. 2). The Western blot data showed that the relative secretion level almost paralleled the total expression level. These results suggest that the signal peptide greatly affected the level of expression and consequently the level of secretion. Northern blot analysis was performed to determine whether the changes in expression level originated in the transcription or the translation

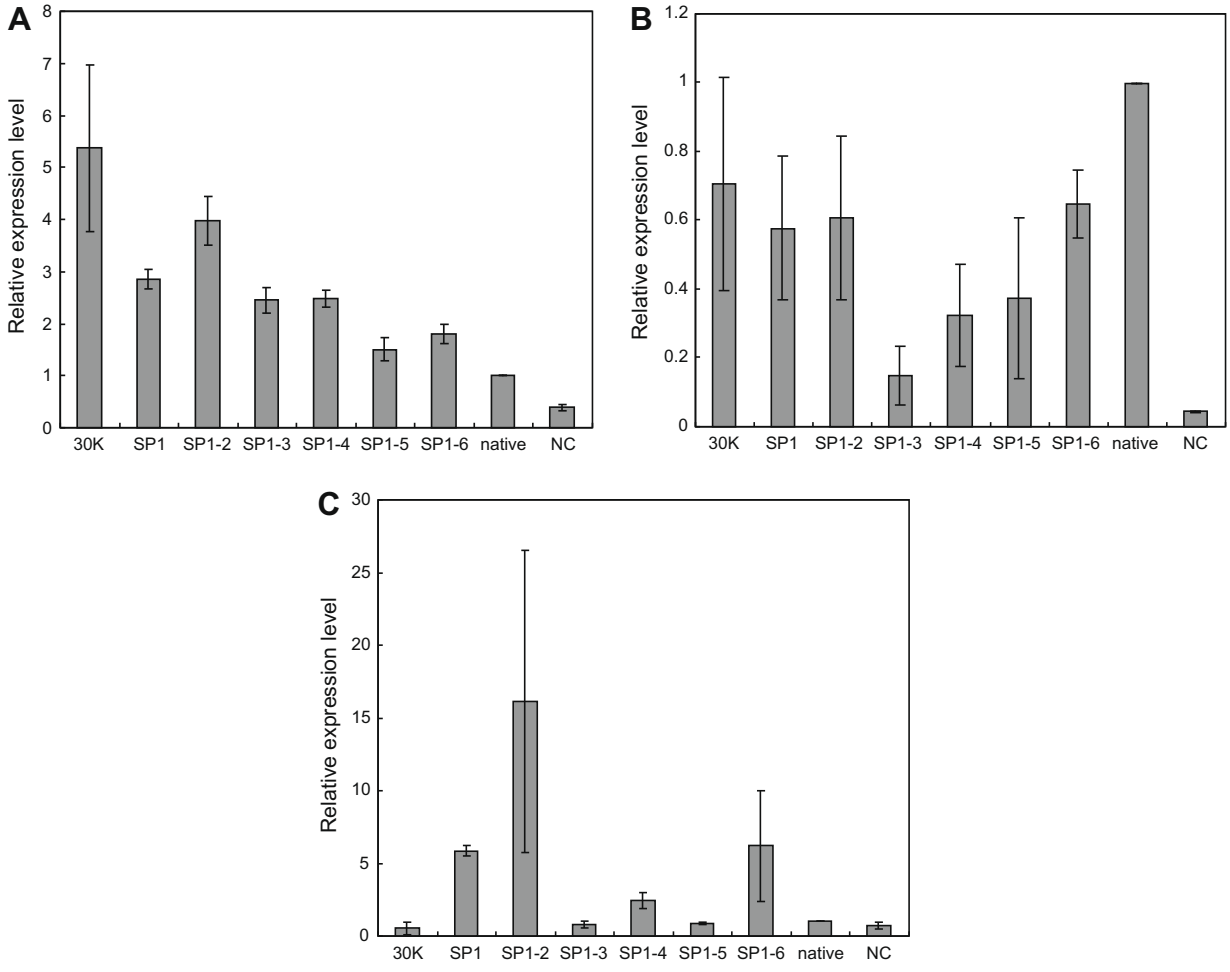


**Fig. 2.** Measurement of total amounts of expressed proteins. Western blot analysis of human IL-4 (A), human IL-13 (B), or human IL-11Rα1(ECD) (C) fused with each signal peptide. Relative expression level of each signal peptide to the native one was measured as described in Materials and methods. Errors are standard deviations of five independent experiments. NC: negative control, which is non-infected cell medium.

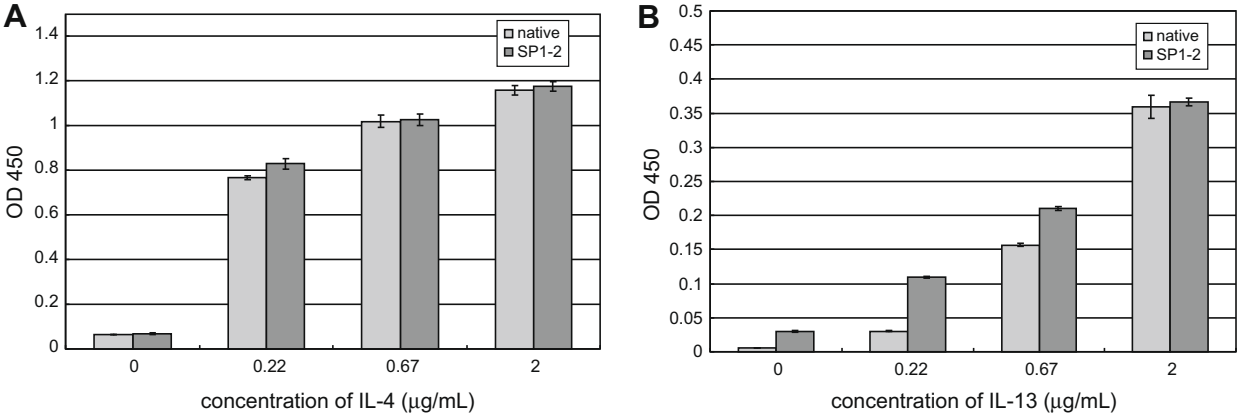


**Fig. 3.** Evaluation of biological activity of expressed cytokines. Sandwich ELISA analysis of human IL-4 (A), human IL-13 (B) fused with SP1–2. The data using native proteins are shown as references.

stage. The mRNA levels of each of the proteins fused with each of the signal peptides were almost identical to each other (data not shown), suggesting that the signal peptide affects the expression level during translation. Therefore, in cases where there was little secretion of the protein fused with a particular signal peptide, targeting to the membrane of the endoplasmic reticulum or insertion of the signal peptide into the membrane of the endoplasmic reticulum was likely to have been insufficient, which might lead to a decrease in the expression level followed by a reduced secretion level.

*Evaluation of biological activity of the expressed cytokines*

To evaluate biological activity of cytokines fused with an engineered signal peptide, the binding assay of the expressed cytokine and its specific receptor was performed by sandwich ELISA. As shown in Fig. 3, IL-4 and IL-13 fused the SP1–2 signal had an identical binding activity for the receptor to their native proteins.

## Conclusion

This study has suggested that polar amino acids in the C-terminal region of a signal peptide play an important role in the secretion of the recombinant protein in BEVS. Compared with the SP1 signal peptide, the SP1–2 signal peptide improved the overall secretion yields of each of the proteins reported here. Fusion of the SP1–2 signal peptide improved the secretion levels of hIL-4 and hIL-13, which each have an α-helix bundle structure, and of hIL-11Rα1(ECD), which has a β-sheet barrel structure. Therefore, the SP1–2 signal peptide might be a universally effective signal in BEVS that is not dependent on the secondary structure of the fused proteins. Finally, the SP1–2 signal peptide will probably be effective in the secretion of recombinant proteins using not only BmN cells, but also other insect cells, such as Sf or Tn cells.

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