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Comparison of recombinant protein expression in a baculovirus system in insect cells (Sf9) and silkworm

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Using a hybrid baculovirus system, we compared the expression of 45 recombinant proteins from six categories using two models: silkworm (larvae and pupae) and an Sf9 cell line. A total of 45 proteins were successfully expressed; preparation of hybrid baculovirus was unsuccessful for one protein, and two proteins were not expressed. A similar pattern of expression was seen in both silkworm and Sf9 cells, with double and multiple bands found in immunoblotting of the precipitate of both hosts. Degraded proteins were seen only in the silkworm system (particularly in the larvae). Production was more efficient in silkworms; a single silkworm produced about 70 times more protein than 10^6 Sf9 cells in 2 ml of culture medium.

Keywords: baculovirus/expression/protein/Sf9/silkworm.

Abbreviations: ADAM, a disintegrin and metalloproteinase; ASB, ankyrin repeat and SOCS box-containing; ATF, activating transcription factor; AZK, α motif and leucine zipper containing kinase; CCR, chemokine (C–C motif) receptor; CD, CD97 molecule; DDR, discoidin domain receptor; DKK, dickkopf; DPP, dipeptidyl-peptidase; ERN, endoplasmic reticulum to nucleus signalling; FGF, fibroblast growth factor; FIGF, c-fos induced growth factor; GPBAR, G protein-coupled bile acid receptor; GPR, G protein-coupled receptor; HTR, 5-hydroxytryptamine (serotonin) receptor; IFN, interferon; IGFBP, insulin-like growth factor binding protein; IL8RB, interleukin 8 receptor β; JNK, c-Jun N-terminal kinase; LGR, leucine-rich repeat-containing G protein-coupled receptor; L3MBTL, lethal (3) malignant brain tumour-like protein; MAP2K3, dual specificity mitogen-activated protein kinase 3; MMP, matrix metallopeptidase; MYST, MYST histone acetyltransferase; PDE4C, phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homologue, Drosophila); PLG, plasminogen; SENP, SUMO1/sentrin specific peptidase; SERPINB, serpin peptidase inhibitor, clade B (ovalbumin), member; SLC, solute carrier family; SPHK, sphingosine kinase; SPPL, signal peptide peptidase-like; STK, serine/threonine kinase; TCF, transcription factor; TXK, tyrosine-protein kinase; UGDH, UDP-glucose dehydrogenase; WNT, wingless-type MMTV integration site; XPGC, DNA repair protein/yeast Rad family protein; ZNF, zinc finger protein.

Many different hosts are used to produce recombinant proteins (*1–3*). The baculovirus expression system is a popular and effective method for large-scale production of vertebrate gene products, because it can express large quantities of vertebrate proteins with appropriate post-translational modifications (*4*). The baculoviruses most commonly used in gene expression studies are *Autographa californica* nuclear polyhedrosis virus (AcNPV), with insect cell lines such as Sf9 or Tn5 as the host and *Bombyx mori* NPV (BmNPV), with silkworm larvae or pupae as the host. These two baculovirus expression systems have different advantages; the AcNPV–insect cells can be cultured with serum-free media, making it easy to purify the products accumulated in the fluids (*5*), whereas the BmNPV–silkworm system has high expression efficiency (*6–8*).

The expression, purification, recovery and function of individual proteins produced by the baculovirus expression system have been described in many papers (*4–10*). Until this present report, there has been no simultaneous, comprehensive or systematic comparison of these proteins. The aim of this study is to demonstrate differences in expression pattern and characteristics among the different types of protein produced by the baculovirus expression system.

AcNPV and BmNPV are restricted in their host range; AcNPV cannot infect the silkworm and BmNPV cannot infect an insect cell line. Thus, comparing gene expression in the two systems has been difficult. However, one study expanded the host range of BmNPV and AcNPV (*9*), and another used firefly luciferase as a reporter expressed by a hybrid baculovirus vector in both an insect cell line and the silkworm (*10*).

Here, we compared expression of recombinant proteins using hybrid baculovirus with an insect cell line

and the silkworm. We studied 45 proteins from six categories including cytokines, G-protein coupled receptors (GPCR), transcription factors, kinases, proteases and 'other' proteins, focusing on the intactness of the expressed protein.

Materials and Methods

Transfer vector

All transfer vectors for the hybrid baculovirus expression system were constructed using the Gateway system (Life Technologies, Carlsbad, CA, USA). A total of 48 genes were prepared as Gateway entry clones from a 'human protein factory' resource (*11*). Other genes were amplified by polymerase chain reaction (PCR) from the cloning vector. Each amplification product was digested by the appropriate restriction enzymes and inserted into the pENTR1A vector. The destination vector was constructed by modifying the pM23 vector (Katakura Industries, Chuo, Tokyo, Japan) by using the Gateway vector conversion system. The construction of the destination vector and the transfer vector is shown in Fig. 1. The transfer vector was constructed by performing the LR recombination reaction with the destination vector and the entry clone (*12*). The transfer vector was designed with a FLAG tag added to the C-terminus.

Hybrid baculovirus and recombinant baculovirus for protein expression

BmNPV (Cpd strain) (*13*) DNA and AcNPV DNA were prepared from the culture medium of an infected BmN cell line and an infected Sf9 cell line, as described by Kondo and Maeda (*9*). The BmNPV DNA was then digested with the restriction endonuclease *Bam*HI. AcNPV DNA and the cleavage fragments of BmNPV DNA were then mixed in TC-100 medium containing lipofectin reagent (Life Technologies). BmN cells were incubated with this lipofectin mixture for 20 h. The transfection solutions were then removed and the cells were cultured for a further 7 days in fresh TC-100 containing 10% foetal bovine serum (FBS). After this incubation period, Sf9 cells were exposed to medium containing hybrid baculovirus for 7 days. The treatment (*i.e.* culture in Sf9 cells after incubation in BmN cells) was repeated six times. This approach allowed infection of the BmN and Sf9 cell lines. The hybrid baculovirus was isolated and the viral titres were measured by endpoint dilution on 96-well plates (*14*).

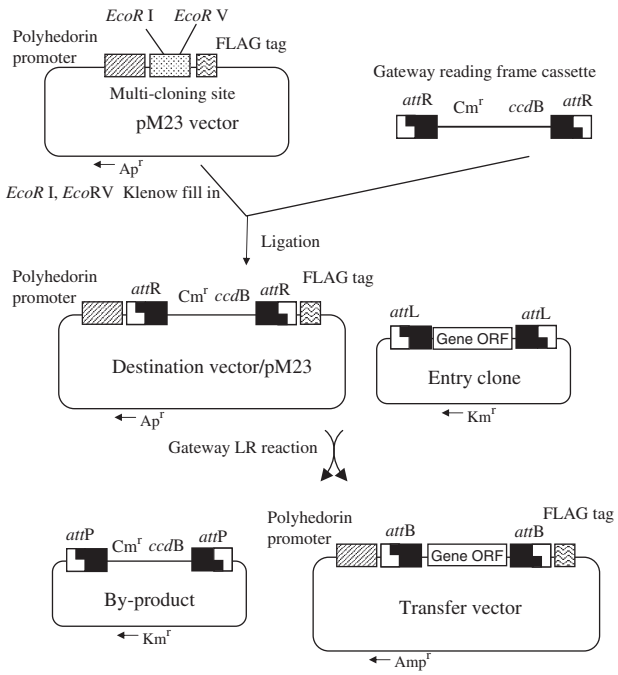

Fig. 1 Construction of transfer vector with Gateway system for baculovirus expression system. The pM23 vector was cut by EcoRI and EcoRV restriction enzymes. The EcoRI site was then filled in with the Klenow fragment of DNA polymerase I using a DNA blunting kit. The destination vector/pM23 was constructed by inserting a Gateway reading frame cassette (RfB) between the blunting sites of pM23. The transfer vector was produced by performing the LR recombination reaction with destination vector/pM23 and an entry clone containing the gene of interest.

The recombinant transfer vector was co-transfected with hybrid baculovirus DNA into the BmN cell line. Recombinant baculovirus was derived from the medium of the BmN cell line after 7 days incubation. This baculovirus was measured in plaque forming units (pfu) by endpoint dilution on 96-well plates (*14*).

Expression proteins

In the case of silkworm expression, the recombinant hybrid baculovirus (5×10^4 pfu/head) was injected into the body cavities of silkworm larvae at the early stage of fifth instar, and into silkworm pupae. The haemolymph was collected at 6 days after infection from silkworm larvae as secretory protein (~0.5 ml haemolymph can be harvested from one larva). Phenylthiourea powder (Wako Pure Chemical Industries, Ltd, Chuo, Osaka, Japan) was added at a final concentration of 16 mM to the collected haemolymph, to prevent melanization. The recovered haemolymph was ultracentrifuged at 100,000*g* for 60 min at 4°C (Beckman Coulter Inc., Fullerton, CA, USA). The infected silkworm larvae and pupae were harvested at 6 days after virus inoculation and stored at −80°C until required for the homogenization step.

For expression in Sf9 cells, 1×10^6 cells/35-mm dish were inoculated with recombinant hybrid baculovirus at a multiplicity of infection of 1 or 5 pfu/cell. Virus was allowed to absorb for 1 h at 27°C. The inoculum was removed and replaced with 2 ml of EX-CELL (Sigma-Aldrich, St Louis, MO, USA). After incubation for 3 days at 27°C, infected cells were scraped from the plate and pelleted by centrifugation at 1,940*g* for 5 min at 4°C. The supernatant was retained as secretory protein and the pellet was stored at −80°C until required for the lysis step.

For homogenization, two frozen infected silkworm larvae were added to 20 ml of homogenization buffer [phosphate buffered saline (PBS) containing 10% glycerol, 5 mM mercaptoethanol, 1 mM phenylmethanesulphonylfluoride (PMSF), 10 mM benzamidine and 16 mM phenylthiourea] and homogenized using an HG30 homogenizer (Hitachi Koki Co., Ltd, Minato, Tokyo, Japan) for 5 min at 4°C; silkworm pupae were homogenized using a Shakemaster (Bio Medical Science, Bunkyo, Tokyo, Japan) under the same conditions, but in 10 ml of homogenization buffer rather than 20 ml. The homogenate was centrifuged at 1,940*g* for 10 min at 4°C to remove cell debris, and ultracentrifuged at 100,000*g* for 60 min at 4°C to separate the soluble and insoluble fractions. The precipitate after ultracentrifugation was suspended in 20 ml of homogenization buffer.

The thawed Sf9 cell pellet was washed and suspended in 1 ml of PBS. The suspension containing the Sf9 cells was then disrupted by ultrasonication and lysed using a pellet mixer (Treff AG, Degersheim, Switzerland). The lysate was then ultracentrifuged at 100,000*g* for 60 min at 4°C; the supernatant was retained as soluble protein and the precipitate as insoluble protein. The precipitate after ultracentrifugation was suspended in 2 ml of PBS buffer.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (*15*) using a 5–20% gradient gel (ATTO Corporation, Bunkyo, Tokyo, Japan). Prepared samples from silkworm and Sf9 cells were heated for 15 min at 60°C with the sample buffer [125 mM Tris–HCl pH 6.8, 4% (w/v) SDS, 20% (w/v) glycerol, 0.01% (w/v) bromophenol blue, 10% (v/v) 2-mercaptoethanol and 8 M urea]. Samples for SDS–PAGE were loaded in each well, with the volume depending on the sample.

Expression of protein was detected by immunoblotting with an anti-FLAG rabbit polyclonal antibody (Sigma-Aldrich), with horse radish peroxidase-labelled anti-rabbit IgG (Beckman Coulter Inc.) as the secondary antibody. Molecular weight was confirmed using a marker made in our laboratory (containing five proteins—24.5, 40, 55, 70 and 110 kDa—purified from recombinant FGF1, MAP2K3, ATF4, STK33 and PLG fused FLAG tag, using a FLAG tag

purification system) as M_1 and Magic Mark XP (Life Technologies) as M_2. Protein expression was visualized using the ECL Plus Western Blotting Detection Kit (GE Healthcare UK Ltd, Piscataway, NJ, USA), and emission signals due to FALG-tagged proteins were detected by exposure of the gel to X-ray film (Fujifilm Corporation, Minato, Tokyo, Japan) for 1 min.

Calculation of protein expression

The exposed X-ray films were scanned using a scanner (GT-8700F, Seiko Epson Corporation, Suwa, Nagano, Japan) and the band intensity at the putative molecular weight was measured by densitometry (CS Analyzer, ATTO Corporation). We determined the amount of each marker (M_1) in the 4 µl load volume as 56.5, 26.3, 16.9, 9.6, 4.4 ng for the 24.5, 40, 55, 70 and 110 kDa markers, respectively. The amounts of expressed protein were calculated by comparison to a standard curve of band intensity of molecular markers (M_1) measured by densitometry on one exposed X-ray film and the amount of protein (Fig. 2).

Results

Table I lists the 48 proteins used to validate the protein expression systems. Protein expression was assessed in a total of six sample types including haemolymph, supernatant of homogenate of pupae, precipitate of homogenate of pupae, medium of Sf9 cells, supernatant of Sf9 cell lysates and precipitate of Sf9 cell lysates. The cytokines were expressed in both silkworm and Sf9 cells, and some expression was seen in all six sample types; however, yield and other characteristics varied between samples (Fig. 3). Four cytokines (WNT-3A protein precursor, DKK2, FIGF and FGF7) were not detected in silkworm haemolymph or medium of Sf9 cells but were detected in silkworm pupae and cell lysates, mainly in the precipitate. This finding suggests that the expressed proteins are aggregated inside the cell and are thus not secreted. The remaining cytokines (PLG, FGF1, IGFBP6 and mouse IFNβ) were expressed in all sample types. Silkworm pupae expressed more insoluble than soluble PLG as well as mouse IFNβ, whereas Sf9 cells expressed more insoluble than soluble proteins for all cytokines. Protein expression patterns were similar for silkworm and Sf9 cells; for example, FGF1, IGFBP6, FGF7 and mouse IFNβ were detected as double or multiple bands. In particular, in the precipitate from silkworm and Sf9 cells, mouse IFNβ showed multiple

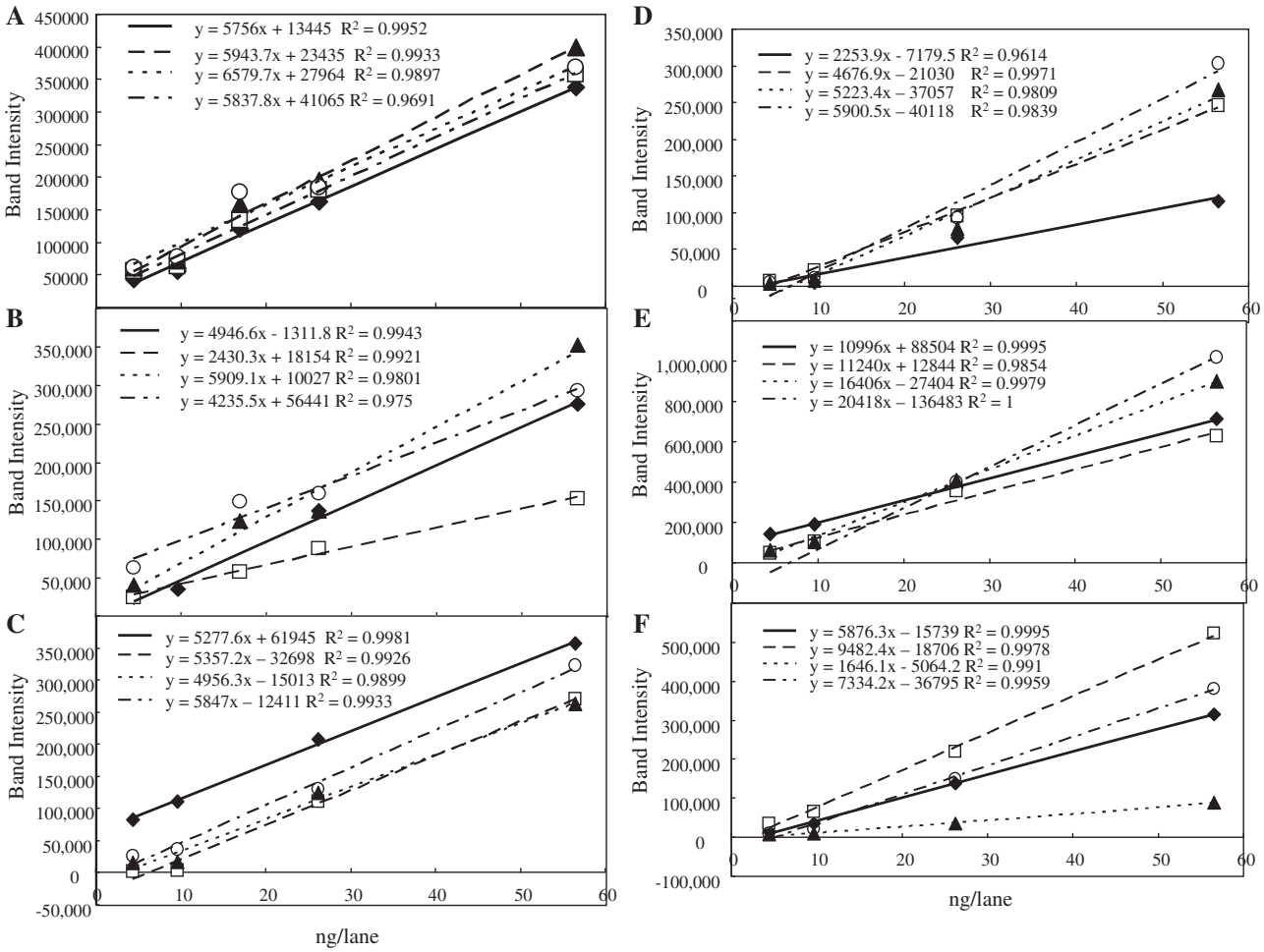

Fig. 2 Standard curves for protein amounts and band intensity of molecular markers (M_1) separated by SDS–PAGE and quantitated by immunoblotting and exposure to X-ray films. (A) Standard curves (4) for eight expressed cytokine proteins. (B) Standard curves (4) for eight expressed GPCR proteins. (C) Standard curves (4) for eight expressed transcription proteins. (D), Standard curves (4) for eight expressed kinase proteins. (E), Standard curves (4) for eight expressed protease proteins. (F), Standard curves (4) for eight expressed other proteins. Filled rhombus, band intensity of each marker (M_1) on one X-ray film (number 1 and 2 for all proteins); open rectangle, band intensity of each marker (M_1) on one X-ray film (number 3 and 4 for all proteins); filled triangle, band intensity of each marker (M_1) on one X-ray film (number 5 and 6 for all proteins); open circle, band intensity of each marker (M_1) on one X-ray film (number 7 and 8 for all proteins).

Table I. Expression levels of 48 proteins shown as per one silkworm and per 1×10^6 Sf9 cells.

			Expression quantity per head (μg)					Expression quantity per 1×10^6cells (μg)					
			Silkworm larvae			Silkworm pupae		Sf9 cell					
				Homogenate		Homogenate			Lysate				
No.	Category	Protein	Haemolymph	Supernatant	Precipitate	Supernatant	Precipitate	Medium	Supernatant	Precipitate	MW (kDa)	FLJ No.	Localization
A-1	Cytokine	WNT-3A protein precursor	0			0	1598	5	0	53	39.4	FLJ31716	Secretory
A-2		PLG	5			16	70	6	0	18	90.6	FLJ93426	Secretory
A-3		FGF1	42			983	200	0	7	0	17.5	FLJ92606	Secretory
A-4		DKK2	0			0	410	0	0	10	28.5	FLJ93094	Secretory
A-5		IGFBP6	319			962	562	34	10	21	25.4	FLJ93653	Secretory
A-6		FIGF	0			0	632	0	0	35	40.5	FLJ93671	Secretory
A-7		FGF7	0			0	229	0	0	32	22.6	FLJ93655	Secretory
A-8		Mouse IFNβ	32			36	610	7	0	21	19.7		Secretory
B-1	GPCR	IL8RB		0	1094	47	402	0	0	4	40.8	FLJ93056	Membrane
B-2		HTR2A		0	699	0	302	0	0	23	52.6	FLJ94830	Membrane
B-3		LGR6		0	706	0	342	0	0	14	99.3	FLJ80387	Membrane
B-4		CD97		0	136	0	9	0	0	13	78.1	FLJ39945	Membrane
B-5		GPR39		0	170	0	46	0	0	0	51.4	FLJ16071	Membrane
B-6		GPBAR1		0	749	0	251	0	0	3	35.3	FLJ16095	Membrane
B-7		GPR125		0	0	0	11	0	0	3	125.3	FLJ38547	Membrane
B-8		G protein-coupled receptor 177isoform 1		170	912	100	248	6	0	10	81.6	FLJ90509	Membrane
C-1	Transcription factor	ATF4		113	432	94	112	0	8	7	38.6	FLJ25022	Inter nuclear
C-2		TCF4		194	366	155	121	0	1	7	71.3	FLJ16304	Inter nuclear
C-3		L3MBTL2		291	1436	131	227	17	25	17	79.1	FLJ31881	Inter nuclear
C-4		GRHL2		285	1232	100	241	0	23	26	71.1	FLJ13782	Inter nuclear
C-5		L3MBTL4		155	866	39	118	0	16	21	71.1	FLJ35936	Inter nuclear
C-6		ZNF233		0	411	0	86	0	0	27	76.8	FLJ38032	Inter nuclear
C-7		ZNF31		0	190	18	65	0	2	13	117.6	FLJ16509	Inter nuclear
C-8		ASB6		0	1148	26	198	3	0	34	47.1	FLJ12942	Inter cellular
D-1	Kinase	JNK3		–	–	–	–	–	–	–	48.1	FLJ42801	Inter cellular
D-2		DDR2		0	90	22	308	7	0	70	96.7	FLJ38656	Inter cellular
D-3		TXK		0	0	0	0	0	0	0	40	FLJ23356	Inter cellular
D-4		AZK		227	1748	75	434	5	4	50	51.6	FLJ31748	Inter cellular
D-5		MAP2K3		846	1856	350	367	30	24	33	36.2	FLJ36519	Inter cellular
D-6		ERN2		0	196	0	58	0	0	3	107.1	FLJ16228	Inter cellular
D-7		SPHK1		0	895	158	254	15	6	20	42.5	FLJ12340	Inter cellular
D-8		STK33		0	1074	270	251	16	9	7	57.8	FLJ35932	Inter cellular
E-1	Protease	SENP6		0	570	0	135	0	2	3	126	FLJ44973	Inter cellular
E-2		DPP III		1	1352	13	375	3	0	32	35.6	FLJ11387	Inter cellular
E-3		SPPL2A		0	650	0	93	0	0	3	58	FLJ91141	
E-4		MMP1		0	863	0	176	4	0	8	53.9	FLJ21165	Secretory
E-5		ADAM12		0	455	28	252	5	0	19	76.8	FLJ45959	Membrane
E-6		Caspase-1		0	1450	85	376	7	0	24	45		Inter cellular
E-7		Caspase-3		1095	467	330	330	21	9	0	31.5		Inter cellular
E-8		Chymase		0	2231	97	610	13	0	13	27.2		Secretory
F-1	Transporter	SLC7A14		0	0	0	163	0	0	10	84.1	FLJ16089	Membrane
F-2		SLC1A2		0	1892	0	393	0	0	9	61.3	FLJ16454	Membrane
F-3	Enzyme	MYST2		995	1281	110	278	0	11	14	70.6	FLJ13828	Inter nuclear
F-4		XPGC		0	950	0	194	0	0	22	102.9	FLJ16464	Inter nuclear
F-5		PDE4C		937	2256	262	411	18	14	31	79.9	FLJ38065	Inter cellular
F-6		UGDH		921	1737	124	367	7	5	27	47.6	FLJ40611	Inter cellular
F-7	Other	SERPINB6		295	1336	159	343	9	6	23	33.2	FLJ16551	Inter cellular
F-8		Influenza A virus hemagglutinin precursor		0	0	0	0	0	0	0	61.9		Membrane

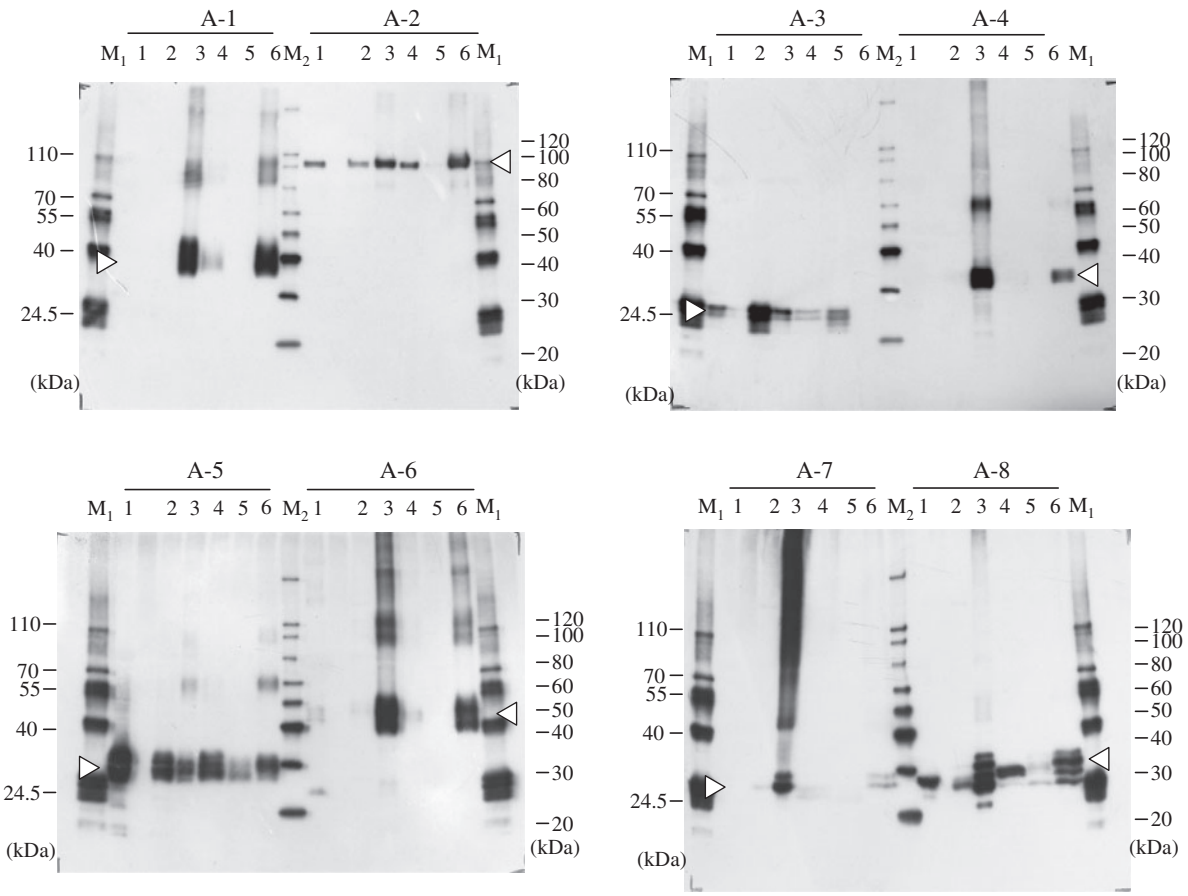

Fig. 3 Expression of proteins (cytokines) detected by immunoblotting. A-1, WNT-3A protein precursor; A-2, PLG; A-3, FGF1; A-4, DKK2; A-5, IGFBP6; A-6, FIGF; A-7, FGF7; A-8, mouse IFNβ; M1, homemade marker for FLAG tag; M_2, Magic Mark XP; lane 1, haemolymph; lane 2, supernatant of homogenate of pupae; lane 3, precipitate of homogenate of pupae; lane 4, medium of Sf9 cell; lane 5, supernatant of Sf9 cell lysate; lane 6, precipitate of Sf9 cell lysate. Arrows indicate the expressed protein at the predicted MW.

bands, because of glycosylation (*16*), and secreted mouse IFNβ showed double bands in both haemolymph of silkworm and in medium of Sf9 cells.

GPCRs are expected to be found in the precipitate after centrifugation because they attach to the membrane of a cell. We confirmed that the GPCRs examined in this study were expressed at the expected molecular weight and that their expression patterns were similar for silkworms and Sf9 cells (Fig. 4). Immunoblotting showed a smear in the high molecular weight region for expressed GPCRs; the expected result for SDS–PAGE of typical membrane proteins (*17*). The GPCRs HTR2A, LGR6, CD97, GPBAR1 were detected in precipitate fractions only, and IL8RB and GPCR 177 isoform 1 were detected as soluble proteins. The precipitate of silkworm larvae homogenate showed degraded protein, especially for IL8RB and GPCR 177 isoform 1.

Transcription factors were detected in silkworm and Sf9 cells (Fig. 5). Three proteins (ZNF233, ZNF31 and ASB6) were expressed mostly as insoluble proteins in both silkworm and Sf9 cells, and to a lesser extent as soluble proteins, particularly in silkworm pupae. The other transcription factors (ATF4, TCF4, L3MBTL2, GRHL2 and L3MBTL4) were expressed as soluble proteins. The amounts of soluble and insoluble expressed proteins in Sf9 cells were similar; however, in silkworm pupae, GRHL2 and L3MBTL4 were mostly expressed as insoluble proteins.

Preparation of a hybrid baculovirus was unsuccessful for the kinase JNK 3. We also found that the kinase TXK was not expressed in any of the sample types (Fig. 6), despite the hybrid baculovirus expressing this protein infecting silkworm and Sf9 cells. MAP2K3 was expressed in all sample types, but the other four kinases were mostly expressed as insoluble proteins (Fig. 6). The expression patterns were similar for silkworm and Sf9 cells, except for STK33, which was expressed by Sf9 cells mostly as a soluble protein.

Proteases were expressed in all samples (Fig. 7), mostly as insoluble proteins; soluble protein was detected only for caspase-3. Degraded protein was detected in both silkworm larvae and pupae (e.g. for SENP, SPPL2A, ADAM12 and caspase-3), but not in Sf9 cells.

The 'other' category of proteins included two transporters and four enzymes (Fig. 8). Influenza A virus haemagglutinin precursor was not expressed in any sample types. The two transporters were expressed in both silkworm and Sf9 cells as insoluble proteins only; SLC7A14 was expressed at its expected molecular weight in silkworm pupae but not in larvae. The four enzymes were expressed in both silkworm and Sf9 cells, although XPGC was expressed only as an insoluble

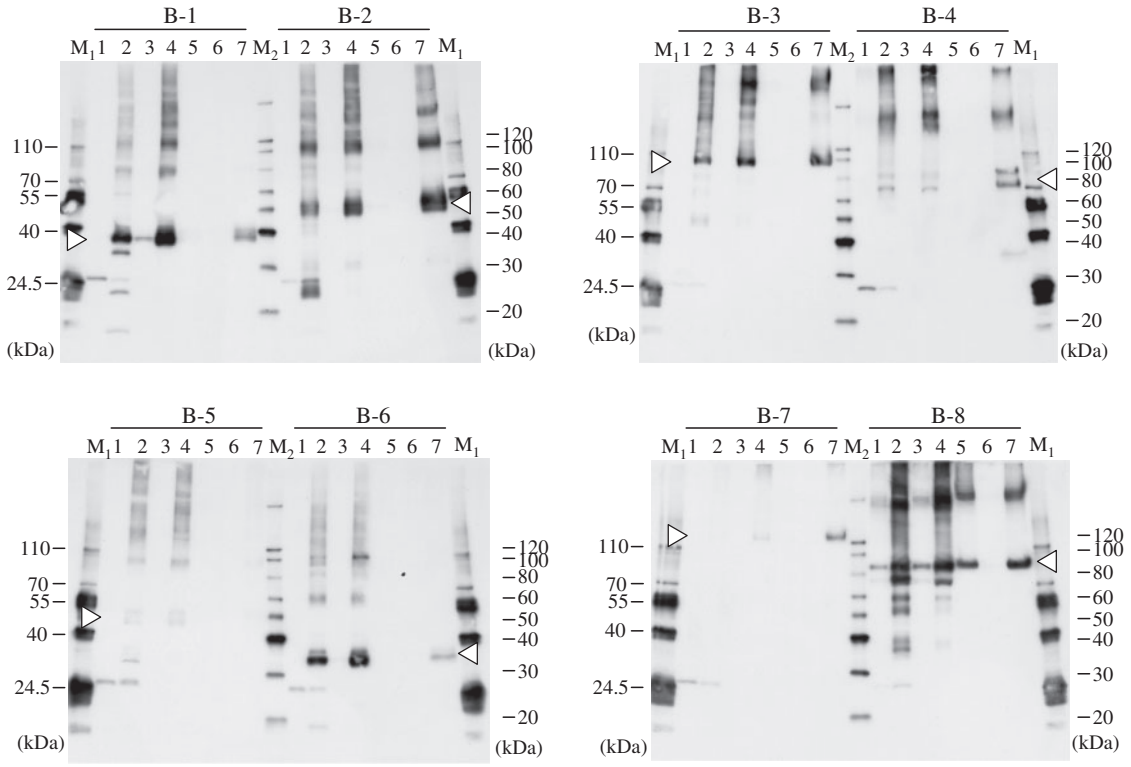

Fig. 4 Expression of proteins (GPCRs) detected by immunoblotting. B-1, IL8RB; B-2, HTR2A; B-3, LGR6; B-4, CD97; B-5, GPR39; B-6, GPBAR1; B-7, GPR125; B-8, G protein-coupled receptor 177 isoform 1; M_1, homemade marker for FLAG tag; M_2, Magic Mark XP; lane 1, supernatant of homogenate of larvae; lane 2, precipitate of homogenate of larvae; lane 3, supernatant of homogenate of pupae; lane 4, precipitate of homogenate of pupae; lane 5, medium of Sf9 cell; lane 6, supernatant of Sf9 cell lysate; lane 7; precipitate of Sf9 cell lysate. Arrows indicate the expressed protein at the predicted MW.

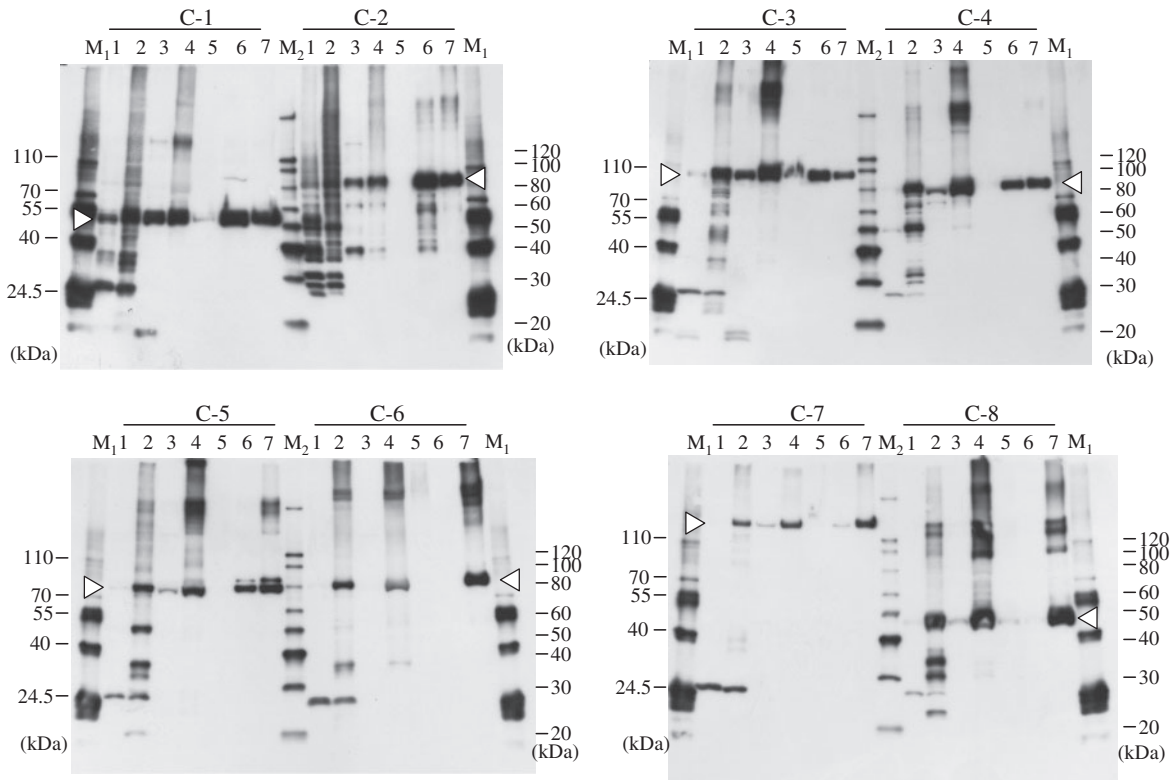

Fig. 5 Expression of proteins (transcription factors) detected by immunoblotting. C-1, ATF4; C-2, TCF4; C-3, L3MBTL2; C-4, GRHL2; C-5, L3MBTL4; C-6, ZNF233; C-7, ZNF31; C-8, ASB6; M_1, homemade marker for FLAG tag; M_2, Magic Mark XP; lane 1, supernatant of homogenate of larvae; lane 2, precipitate of homogenate of larvae; lane 3, supernatant of homogenate of pupae; lane 4, precipitate of homogenate of pupae; lane 5, medium of Sf9 cell; lane 6, supernatant of Sf9 cell lysate; lane 7, precipitate of Sf9 cell lysate. Arrows indicate the expressed protein at the predicted MW.

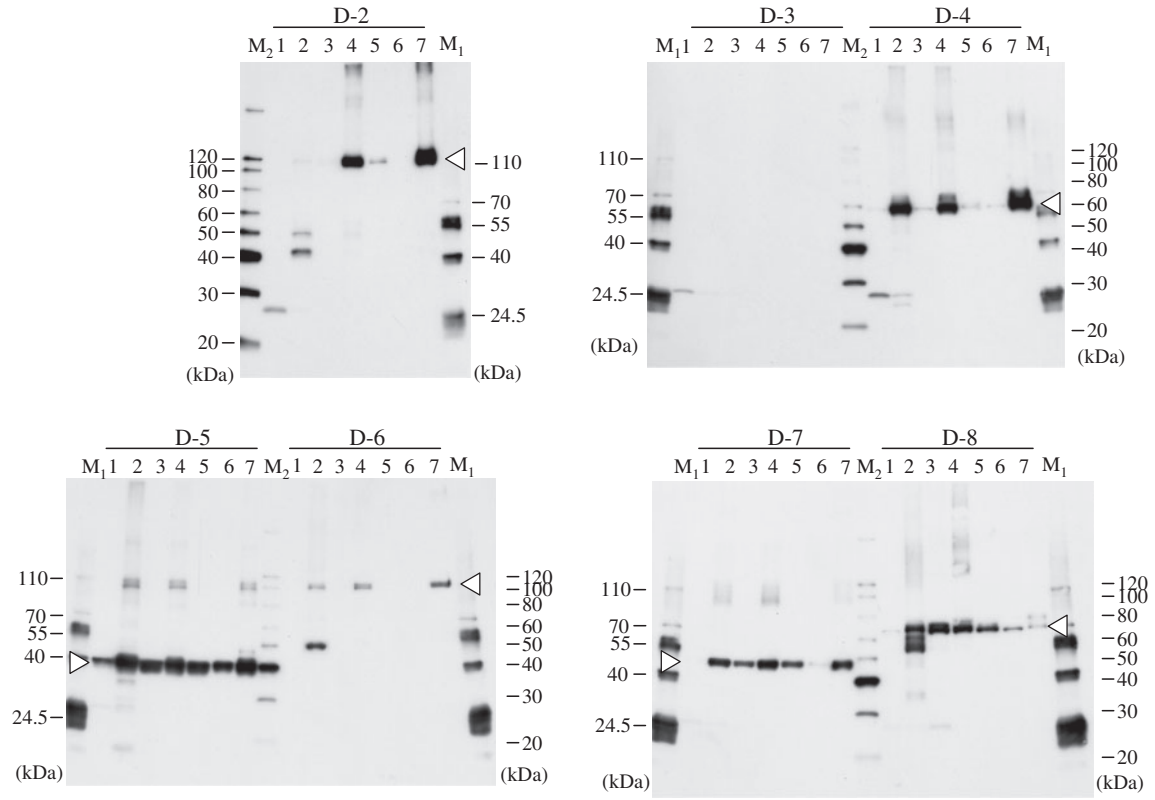

Fig. 6 Expression of proteins (kinases) detected by immunoblotting. D-2, DDR2 4; D-3, TXK; D-4, AZK; D-5, MAP2K3; D-6, ERN2; D-7, SPHK1; D-8, STK33; M_1, homemade marker for FLAG tag; M_2, Magic Mark XP; lane 1, supernatant of homogenate of larvae; lane 2, precipitate of homogenate of larvae; lane 3, supernatant of homogenate of pupae; lane 4, precipitate of homogenate of pupae; lane 5, medium of Sf9 cell; lane 6, supernatant of Sf9 cell lysate; lane 7, precipitate of Sf9 cell lysate. Arrows indicate the expressed protein at the predicted MW.

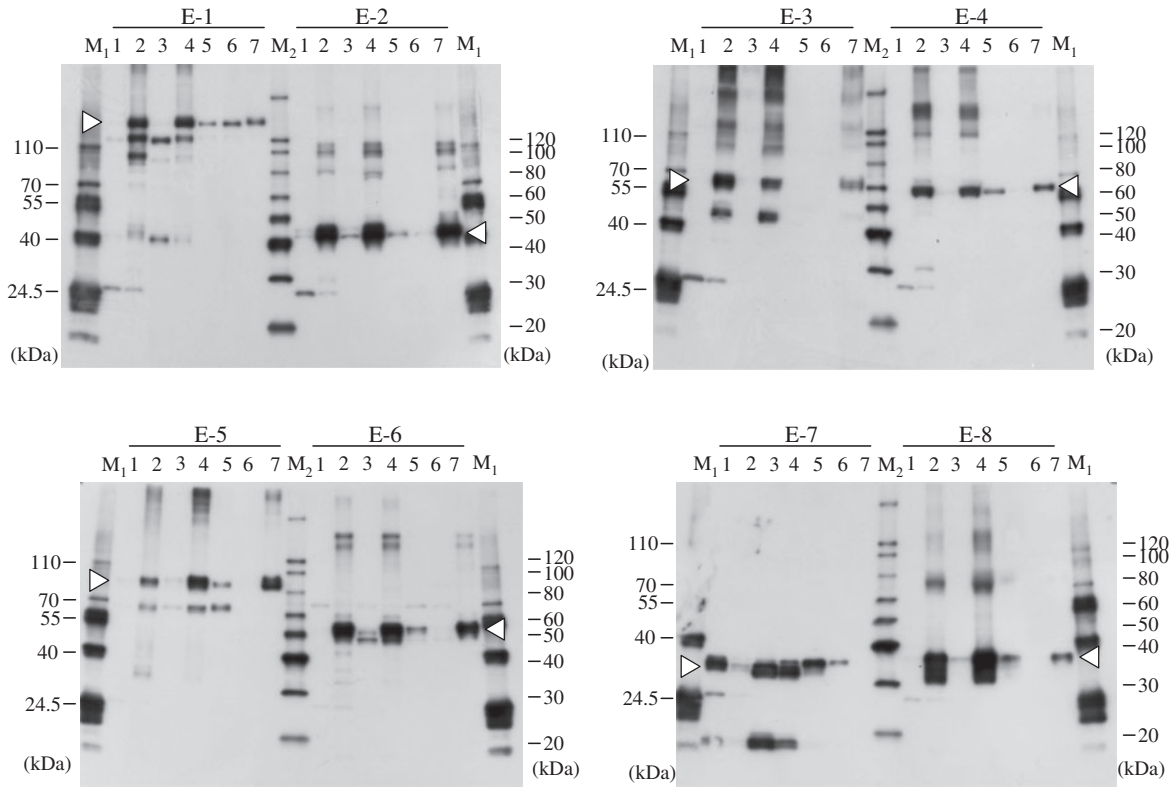

Fig. 7 Expression of proteins (proteases) detected by immunoblotting. E-1, SENP6; E-2, DPP III 4; E-3, SPPL2A; E-4, MMP1; E-5, ADAM12; E-6, aspase-1; E-7, Caspase-3; E-8, Chymase; M_1, homemade marker for FLAG tag; M_2, Magic Mark XP; lane 1, supernatant of homogenate of larvae; lane 2, precipitate of homogenate of larvae; lane 3, supernatant of homogenate of pupae; lane 4, precipitate of homogenate of pupae; lane 5, medium of Sf9 cell; lane 6, supernatant of Sf9 cell lysate; lane 7, precipitate of Sf9 cell lysate. Arrow indicate the expressed protein at the predicted MW.

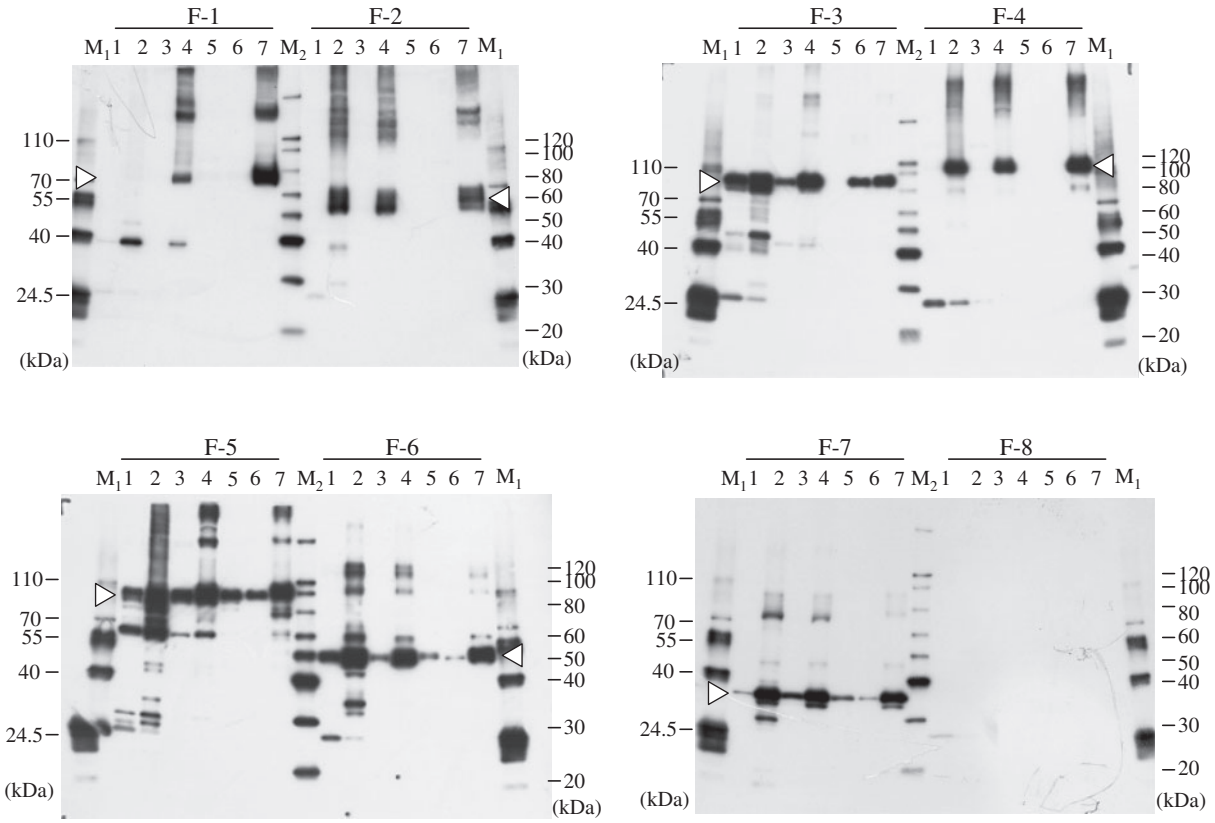

Fig. 8 Expression of proteins (others) detected by immunoblotting. F-1, SLC7A14; F-2, SLC1A2; F-3, MYST2; F-4, XPGC; F-5, PDE4C; F-6, UGDH; F-7, SERPINB6; F-8, Influenza A virus hemagglutinin precursor; M_1, homemade marker for FLAG tag; M_2, Magic Mark XP; lane 1, supernatant of homogenate of larvae; lane 2, precipitate of homogenate of larvae; lane 3, supernatant of homogenate of pupae; lane 4; precipitate of homogenate of pupae; lane 5, medium of Sf9 cell; lane 6, supernatant of Sf9 cell lysate; lane 7, precipitate of Sf9 cell lysate. Arrows indicate the expressed protein at the predicted MW.

protein. The serpin peptidase inhibitor, clade B (ovalbumin) member 6 (SERPINB6) was expressed in all sample types, but mostly as an insoluble protein.

Table I shows the amounts of expressed protein per silkworm larva, pupa, and 1×10^6 Sf9 cells. The protein produced by one silkworm was about 70 times greater than the production by 1×10^6 Sf9 cells.

Discussion

Here, we characterized the expression of 45 recombinant proteins from six different categories that were extracted from silkworm larvae, silkworm pupae and an Sf9 cell line using hybrid baculovirus. To allow a comparison of protein expression between silkworm and cultured cells we calculated the amounts of protein in one silkworm or 1×10^6 cells Sf9 cells according to the method described by Steve (*18*).

We successfully expressed 45 out of a total of 48 proteins; we were unable to generate a hybrid baculovirus for the kinase JNK 3, and two proteins (TXK and influenza A virus haemagglutinin precursor) were not expressed. The yield, solubility and intactness of expressed proteins are thought to depend on the protein type; however, we found no relationship between these expression characteristics and protein type. In general, the protein expression patterns were similar for proteins extracted from silkworm and Sf9 cells; insoluble proteins in the precipitate were found as double and multiple bands by immunoblotting. However, proteins extracted from silkworm, particularly the larvae, tended to be more degraded than those extracted from Sf9 cells. It is possible that protease from the silkworm caused degradation of the expressed proteins following homogenization of the silkworms. The difference in the amount of degradation between the larve and pupae might be due to the presence of digestive enzymes in the midgut of the larvae; the midgut degenerates during metamorphosis in silkworm pupae. We observed limited degradation of proteins expressed by the Sf9 cells, suggesting that the level of proteases in these cells is negligible.

The study clearly showed that the silkworm has advantages over Sf9 cells in terms of total yield of expressed protein. However, the level of expression of soluble protein was higher in Sf9 cells than in the silkworm for all proteins except the membrane proteins. This is most likely because of the greater level of complexity of the biological systems in silkworms compared to those in Sf9 cells. In the baculovirus expression system, expression of proteins is transiently induced by a strong polyhedrin promoter. It is possible that the quality control of protein expression with chaperones cannot be properly regulated. For example, in a separate study, we found that improving the promoter (*vp39* promoter with HR3) affected the

solubility of recombinant protein in the baculovirus system (*19*). When the *vp39* promoter with HR3 was used, the aggregation of foreign proteins expressed by baculovirus expression system was markedly decreased. However, the quantity of expressed protein was lower from this promoter than from the polyhedrin promoter.

We did not attempt to investigate the activities or functions of each recombinant protein produced in this study. There is no doubt that measurement of protein activity reflects the yield of expressed protein. However, it is not possible to measure the activity of many different types of proteins simultaneously because different proteins require different measurement methods. The purpose of this study was to determine a standard measurement system that allows the yield of different proteins to be compared.

Our results show that the baculovirus expression system using silkworm can produce recombinant proteins on a large scale, provided that the expressed proteins are produced in a soluble form. Detergents could be used to increase the yield of soluble proteins, provided that protein activity is maintained. This approach could be useful, for example, in producing proteins for use as antigens, for X-ray crystallography and for drug discovery. The Sf9 cell expression system is a universal and balanced system, because it allows the expression of mainly soluble proteins, and it is the system of choice if quantity is not an issue.

The information derived from this study should be verified for individual proteins. In summary, here we suggest the optimal expression system according to application and whether the study is small or large scale.

Conflict of interest
None declared.

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