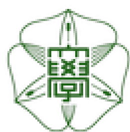

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Efficient preparation of human and mouse CD1d proteins using silkworm baculovirus expression system

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Highlights

- Recombinant human and mouse CD1ds were prepared at high purity using a silkworm-baculovirus expression system.
- Single-chain human CD1d-β2m was expressed at a 10-fold higher yield and exhibited higher thermal stability than the co-expressed human CD1d and β2m.
- The silkworm-baculovirus expression system is useful for the preparation of CD1d.

Abstract

CD1d is a major histocompatibility complex (MHC) class I-like glycoprotein and binds to glycolipid antigens that are recognized by natural killer T (NKT) cells. To date, our understanding of the structural basis for glycolipid binding and receptor recognition of CD1d is still limited. Here, we established a preparation method for the ectodomain of human and mouse CD1d using a silkworm-baculovirus expression system. The co-expression of human and mouse CD1d and β2-microglobulin (β2m) in the silkworm-baculovirus system was successful, but the yield of human CD1d was low. A construct of human CD1d fused with β2m via a flexible GS linker as a single polypeptide was prepared to improve protein yield. The production of this single-chained complex was higher (50 μg/larva) than that of the co-expression complex. Furthermore, differential scanning calorimetry revealed that the linker made the CD1d complex more stable and homogenous. These results suggest that the silkworm-baculovirus expression system is useful for structural and biophysical studies of CD1d in several aspects including low cost, easy handling, biohazard-free, rapid, and high yielding.

Keywords: CD1d; Secretory expression; Major histocompatibility complex class I; Differential scanning calorimetry; Silkworm; Baculovirus

Introduction

CD1 family proteins are major histocompatibility class (MHC) I-like molecules; however, they present lipid-based antigens instead of peptides [1]. The CD1 family has five proteins, CD1a, -b, -c, -d, and -e, in humans, and two proteins, CD1d1 and CD1d2, in mice [2]. Ligands of CD1 proteins range from self-lipids, common in mammalian organisms, to foreign lipids specific to microorganisms. CD1 proteins are classified into three groups: CD1a, -b, and -c (group 1), CD1d (group 2), and CD1e (group 3). Group 1 and group 2 CD1 proteins are recognized by T cell receptors (TCRs), whereas CD1e is not recognized by TCRs and its function remains unclear [3]. CD1d presents lipid-based antigens to immune cells, including natural killer T (NKT) cells and γδ T cells [4–6]. CD1d has a similar structure to MHC class I molecules [7], composed of an immunoglobulin fold and a hydrophobic ligand binding pocket formed by two α-helices and one β-sheet, which has a role for presenting various lipids, such as phospholipids,

bacterial diacylglycerol, parasite lipophosphoglycan [8–11]. NKT cells serve an important role in transmitting innate immune signals to the adaptive immune system [12]. Furthermore, the CD1d mediated NKT cell immune system is involved in cancer proliferation [13], auto-immune disease [14], allergy [15], and infectious diseases [16]. The post-translational modification in insect cells is similar to that of mammalian cells; thus, many proteins can be expressed in their native form. Although the type of *N*-linked glycosylation differs slightly from that of mammalian proteins, target protein function is largely unaffected [17]. The pauci-mannose or high-mannose type *N*-glycans modified by insect cells [18] are unique and have advantages for structural studies. An insect cell expression system is predominantly used to prepare the CD1d ectodomain for experiments that require high purity protein, as described in the X-ray crystallographic study [19,20]. The silkworm baculovirus expression system provides high titers of P1 virus, enables rapid protein expression, and has been established as a powerful tool for recombinant protein production [21]. In the silkworm-baculovirus expression system, although the experimental steps producing recombinant baculovirus are the same as that of the insect cell expression system, the type of baculovirus used, *Bombyx mori* nuclear polyhedrosis virus (BmNPV), is different from that of the insect cell expression system [17]. We succeeded in expressing the cell surface receptor, human killer-cell immunoglobulin-like receptor (KIR2DL1) [22], and G protein-coupled receptor (GPCR), human nociceptin receptor [23], using the silkworm-baculovirus expression system. The functions of expressed proteins were confirmed by surface plasmon resonance (SPR) for KIR2DL1, and the [^{35}S]GTPγS-binding assay for the nociceptin receptor. Here, we applied this system for the expression of human CD1d protein and assessed the biophysical aspects of the expressed proteins.

Here, we report a new preparation method for human and mouse CD1d ectodomain using the silkworm-baculovirus expression system and characterized the biophysical aspects of the prepared proteins. The ectodomain of human and mouse CD1d was co-expressed with cognate β2-microglobulins (β2m), secreted into hemolymph of silkworms, and purified to be suitable for biophysical experiments. To increase the stability of human CD1d, the heavy chain of human CD1d was fused with human β2m via a flexible linker motif, and they were expressed as a single polypeptide chain (schCD1d). schCD1d protein was expressed in silkworms and was purified in the same

manner as for the CD1d-β2m co-expression. The protein yield of schCD1d was ten times higher than that of the non-fused human CD1d heterodimer. The differential scanning calorimetry (DSC) analysis showed that schCD1d was more stable and homogenous than non-fused human CD1d heterodimer.

Materials and methods

Materials

Silkworm (*B. mori*, Kinshu × Showa race) was purchased from Ehime sanshu (Ehime, Japan) and was cultivated with an artificial diet (Nosan Corporation, Kanagawa, Japan) at 25°C. Human CD1d gene was purchased from Kazusa DNA clone (product ID: FHC20648). Human β2m … Mouse CD1d1 and mouse β2m genes were codon optimized and chemically synthesized (Eurofins Genomics K.K., Tokyo, Japan).

Construction of CD1d expression vector

For the co-expression of human CD1d-β2m and mouse CD1d-β2m, the genes encoding CD1d and β2m were inserted under the polyhedrin and p10 promotors in the pFastBac-Dual plasmid (Invitrogen, CA, United States), respectively. Polymerase chain reaction (PCR) was employed to amplify the DNA fragments of the human CD1d ectodomain (GenBank accession no. AB590619.1, 1-295aa) and mouse CD1d1 ectodomain (GenBank accession no. NM_007639.3, 1-297aa) with a C-terminal hexahistidine tag, and human β2m (GenBank accession no. V00567.1, 1-119aa) and mouse β2m (Gen Bank accession no. X01838.1, 1-119aa). The DNA fragments were digested by restriction enzymes, NotI and HindIII for human CD1d, BamHI and NotI for mouse CD1d1, KpnI and NcoI sites for human β2m, and XhoI and NheI for mouse β2m. This was followed by ligation with pFastBac-Dual plasmid. Concerning single chain CD1d (schCD1d), human β2m and CD1d were connected by a 12-mer GS linker (GGGGSGSGGGGS) and a hexahistidine tag was fused at the C-terminal end of the CD1d gene. The schCD1d gene was amplified by PCR and subcloned into the pFastBac1 plasmid (Invitrogen) under the polyhedrin promotor using NotI and HindIII sites. The *Escherichia coli* DH5α competent cells were transformed with the ligation products and were cultured on Luria-Bertani agar plates containing ampicillin. The resultant colony was inoculated into 2xYT medium and cultured for 16 hours. The plasmid was isolated

from the cultured cells and purified by a plasmid mini-prep kit (QIAGEN, Hilden, Germany). The sequence of the plasmid was checked by an ABI 3100 sequencer (Applied Biosystems, CA, United States.).

Preparation of recombinant bacmids and recombinant virus

The subcloned pFastBac-dual plasmid was introduced into the *E. coli* BmDH10Bac strain, which has viral cysteine protease- and chitinase-deficient BmNPV bacmid DNA and the helper plasmid pMON7124 [21]. Transposition of the gene from the pFastBac Dual plasmid to the BmNPV bacmid was confirmed by PCR and the recombinant bacmid DNA was purified with a Plasmid Midi Kit (QIAGEN). The purified BmNPV bacmid DNA was mixed with DIMRIE-C reagent (Invitrogen), and was incubated at room temperature for 45 minutes. The resulting mixture was injected into the dorsal region of fifth instar larvae, using a syringe with a 26-gauge needle. After 6 days rearing, hemolymph of silkworm larvae was extracted by puncturing tiny holes at the middle of the body using a syringe with a 26-gauge needle, and 0.5% sodium thiosulfate was added to avoid melanization. The collected hemolymph was centrifuged at 5,000 ×g for 30 min to remove hematocytes, and the supernatant was collected. The collected hemolymph was centrifuged again at 40,000 ×g for 30 min to precipitate the recombinant virus. The viral precipitate was resuspended in 0.4 ml of PBS per larva and frozen with liquid nitrogen and stored in a freezer until use.

Protein expression in silkworm

A syringe was dipped into the virus suspension; then, fifth instar silkworm larvae were infected by piercing with the syringe. After rearing, hemolymph of the silkworm larvae was extracted by piercing and 0.5% sodium thiosulfate was added. Then, it was centrifuged at 40,000 ×g for 30 min to remove particles. Saturated ammonium sulfate was added up to target concentrations and incubated for 1 h at 4°C to precipitate the proteins. The expression of CD1d proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using HisProbe-HRP (Thermo Fisher Scientific, MA, USA).

Purification of CD1d-β2m complex

The hemolymph which hematocyte was removed was brought to a 60% ammonium sulfate saturation and centrifuged for 30 min at 20,000 ×g. The precipitate was resuspended in resuspension buffer (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, and 20 mM Imidazole), then applied to a HisTrap HP column (GE Healthcare, IL, United States) equilibrated with resuspension buffer. Consequently, the column was washed with resuspension buffer, and the bound protein was eluted with a linear gradient of 5–30% (A: B, v/v) buffer A (20 mM Tris-HCl at pH 8.0, 100 mM NaCl) and B (20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 400 mM Imidazole). Each fraction was analyzed by SDS–PAGE with Coomassie staining and western blotting. The fractions containing both CD1d and β2m were concentrated by ultrafiltration with Amicon Ultra (Merck Millipore, MA, USA) and purified by size exclusion chromatography using a Superdex 200 16/60 column (GE Healthcare) with buffer A.

Digestion of N-linked glycans by Endoglycosidases

CD1d proteins were digested by endoglycosidase after 10 minutes denaturing with 0.5% SDS at 95°C. The digestion was performed 1 h or overnight by Endo H_f (New England Biolabs, MA, USA) or PNGase F (New England Biolabs) at 37°C. The digested proteins were analyzed by SDS-PAGE.

Circular dichroism (CD) analysis

CD spectra were recorded on a JASCO J-820 CD spectrometer (JASCO Corporation, Tokyo, Japan). Far-UV CD measurements were performed with 200 nM of each protein in Tris-HCl (pH 8.0), 100 mM NaCl, using a 1 mm pathlength quartz cuvette and a bandwidth of 1 nm. Spectra were accumulated four times.

Differential scanning calorimetry (DSC) analysis

The molar specific heat at constant pressure of CD1d proteins was measured using a differential scanning calorimeter (MicroCal VP-Capillary DSC system; Malvern, Worcestershire, UK). All calorimetric scans were performed in a buffer composed of 10 mM HEPES-NaOH (pH 7.4) and 100 mM NaCl. Protein samples at 0.1–0.2 mg/ml were

heated from 20°C to 90°C at a scanning rate of 60°C/hour. The resultant DSC curves were normalized by subtracting the corresponding buffer baseline as the reference and thereby by using protein concentration, and the data were processed using the non-two-state model fitting with Origin software (OriginLab, Corporation, Northampton, MA).

Results

Co-expression of human CD1d and β2m (hCD1d)

The extracellular domains of human CD1d and human β2m were co-expressed by the silkworm-baculovirus expression system (**Fig. 1**). To assess the expression level of the hCD1d, hemolymph of virus-infected silkworms was collected 4-6 days after infection and subjected to SDS-PAGE and western blotting (**Supplementary Fig. 1A**). The hCD1d was expressed 4 days after virus infection and the expression level gradually increased as time progressed. Although the molecular weight calculated from the amino acid sequence of hCD1d heavy chain is 32.7 kDa, the heavy chain bands that appeared in western blotting were close to 40 kDa. This difference is due to N-linked glycosylation and details are described in next session.

The BmNPV purified from the hemolymph was used for subsequent viral infection. The hemolymph was collected 5 days after the infection because the yield of 6 days had a greater variation than 5 days due to the death of 10-30% silkworm larvae (data not shown). Since the silkworm hemolymph was highly viscous due to abundant endogenous proteins, the collected hemolymph was precipitated by ammonium sulfate prior to Ni-affinity chromatography. The concentration of ammonium sulfate suitable for sample precipitation was checked using 20-80% saturated ammonium sulfate (**Supplementary Fig. 1B**). The hCD1d protein was precipitated in 60% saturated ammonium sulfate. The precipitant was resuspended in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and followed by Ni-affinity chromatography (**Fig. 2A, B**). CD1d and β2m were eluted together when the concentration of the imidazole reached 60 mM in Ni-affinity chromatography. Since silkworm endogenous proteins remain in the collected fractions, the fractions containing CD1d and β2m were subsequently purified by size exclusion chromatography (**Fig. 2C, D**). The hCD1d protein was eluted as a single peak with size exclusion chromatography and few other proteins were observed in the

eluted fractions. The average yield of purified protein was 5 μg per larva.

Preparation of single chain human CD1d-β2m complex (schCD1d)

The CD1d and β2m complex was not sufficiently recovered for further structural studies, such as x-ray crystallography, when they were co-expressed in silkworms. Single chain MHC class I molecules have been developed to improve stability and decrease dependency on chaperone assistance [25–27]. It has been reported that efficient expression of single chain CD1d-β2m can be attained using Fc-fusion and the lentiviral expression system [28]. Thus, CD1d and β2m were genetically connected by a 12-mer GS linker (GGGGSGSGGGGS) and expressed as a single polypeptide chain (schCD1d) (**Fig. 1**) to increase the stability of CD1d-β2m complex and improve the yield. The DNA encoding schCD1d was introduced into pFastBac1, expressed in silkworms using the recombinant virus vector and purified from hemolymph in the same manner as hCD1d. For the first step of schCD1d purification, schCD1d protein was completely precipitated with 60% saturated ammonium sulfateschCD1d (**Supplementary Fig. 1C**). The precipitant was resuspended and purified by Ni-affinity chromatography (**Fig. 3A, B**) and subsequent size exclusion chromatography (**Fig. 3C, D**). The schCD1d proteins were eluted when the concentration of the imidazole reached 60 mM in Ni-affinity chromatography. Silkworm endogenous proteins that were contained in fractions with schCD1d were removed by size exclusion chromatography. schCD1d was obtained as a single band, revealed by SDS-PAGE (**Fig. 3D**). The elution position indicates a molecular weight of 36 kDa (**Supplementary Fig. 2B**), consistent with that of schCD1d, suggesting that the schCD1d protein prepared here adopts native folds and forms a correct dimer complex without forming high-molecular weight inter-chain oligomers. The yield of schCD1d was 50 μg per larva and it was ten times higher than that of CD1d co-expression. Since CD1d protein is unstable without β2m, the significant improvement in protein yield seems to be caused by improved stability of CD1d, facilitated by the linked β2m protein.

Preparation of mouse CD1d and β2m (mCD1d)

The extracellular domain of mouse CD1d and (1-297 aa) and mouse β2m (1-119 aa) were co-expressed in same manner as human CD1d. Briefly, viral DNA or virus

particles containing mouse CD1d and β2m DNA sequences were transfected into silkworm larvae. The mCD1d in hemolymph was precipitated by 60% saturated ammonium sulfate (**Supplementary Fig. 1D**). The mCD1d was purified by Ni-affinity chromatography and subsequently, size exclusion chromatography (**Fig. 4A-D**). The yield of mCD1d was 50 μg per larva.

N-linked glycosylation of CD1d

There are four potential N-linked glycosylation sites, Asn38, Asn60, Asn126 and Asn181, in human CD1d heavy chain and Asn38, Asn60, Asn128 and Asn183, in mouse CD1d heavy chain. Previous reports clarified that crystal structures of human and mouse CD1d-β2m proteins expressed in insect cells have N-linked glycosylation at least in Asn38 and Asn60 of human CD1d [24] and Asn38, Asn60 and Asn183 of mouse CD1d [29], thus it raise the possibility that the CD1d expressed in silkworm also receive sugar modifications in the same manner. To examine N-linked glycosylation of CD1d proteins prepared from silkworm expression system, each CD1d protein was treated with Endo Hf or PNGase F, which eliminate the N-linked sugars differently, and subjected to SDS-PAGE. After enzyme digestion, the bands of CD1d proteins were down shifted from the original position. The resultant band positions corresponded to the estimated molecular weights (**Fig. 5**). Treatment with PNGase F moved the band to the significantly lower position than treatment with Endo Hf, since PNGase F digests the closer site from protein than Endo Hf does. These results clearly indicate the presence of N-linked glycosylation in CD1d expressed in silkworm.

Circular dichroism (CD)

In order to evaluate the overall structure of CD1d proteins, far-UV CD spectra were measured (**Fig. 6A**). All spectra have a substantial negative band near 218 nm, indicating that a β-sheet is the main component of CD1d that corresponds to the immunoglobulin fold of CD1d and β2m. Within the negative band near 218 nm, a small negative band was observed near 210 nm in all spectra. This small negative band near 210 nm implies the presence of an α-helix, which is a common feature of ligand binding pockets in major histocompatibility class I proteins. The deep trough at 218 nm is more

consistent with MHC-I-peptide complex than MHC-I without peptide [30], suggesting that CD1d is a native lipid-containing structure. The CD spectrum of schCD1d was essentially the same as hCD1d, supporting the idea that schCD1d is correctly folded as is the native CD1d-β2m heterodimeric complex. The CD spectrum of mCD1d was also the same as hCD1d, suggesting that the overall structures of human and mouse CD1d that share high conserved sequences, even when prepared in a silkworm expression system, are essentially the same.

Differential scanning calorimetry (DSC)

To evaluate the effect of the modification on thermal stability, CD1d proteins were analyzed by DSC (**Fig. 6B**). The melting temperature of schCD1d was slightly higher than hCD1d (schCD1d: 62.7°C, hCD1d: 61.5°C), suggesting that schCD1d has higher thermal stability. In addition, the melting curve was sharper than that of hCD1d, which may reflect that schCD1d is more homogenous than the non-fused CD1d-β2m complex. The thermal stability of schCD1d seems to be acquired by the linker between CD1d and β2m, since the flexibility of schCD1d is restricted to some extent by the linker. The significantly increased yield by single-chain modification may be involved in the improvement of CD1d stability. The melting temperature of mCD1d was 61.7°C, which was almost the same value as hCD1d. However, the peak was narrow, suggesting that a more uniform population than hCD1d.

Discussion

In this study, the human and mouse CD1d ectodomain was mass expressed in the hemolymph of silkworm larvae by using a baculovirus vector. The hCD1d was expressed by transfection with viral DNA or by inoculation of silkworm larvae with the amplified virus. The hCD1d was successfully purified from hemolymph to a high purity that can be used for physicochemical analysis; however, the yield was modest compared with those of other cell surface protein ectodomains, such as IL_4-Rα (55 μg per larva) expressed in a silkworm-baculovirus expression system [31].

This study showed that the connection between the CD1d heavy and light chains contributes to improving the expression level and stability of CD1d generated in silkworm expression systems. In one study, a full-length antibody expression was performed in a

silkworm expression system, using molecular chaperones to improve the yield of heterodimers [32]. The β2m plays the role of a chaperone for MHC-I molecules. Thus, the chaperones ability to improve protein yield may be dependent on its efficient association with the heavy chain via single-chain formation.

Conclusion

We established a new preparation method for human and mouse CD1d-β2m fusion proteins using a silkworm-baculovirus expression system and characterized the prepared proteins. The CD analysis showed that CD1d proteins were mainly composed of β-sheets that correspond to the Ig-fold and α-helix that forms the ligand binding pocket. Furthermore, the expression level and thermal stability of schCD1d were increased compared to non-fused CD1d-β2m. This study clearly shows that the silkworm-baculovirus expression system is useful to produce CD1d proteins in terms of yield and quality.

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References

[1] S.A. Porcelli, R.L. Modlin, The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids., Annu. Rev. Immunol. 17 (1999) 297–329. https://doi.org/10.1146/annurev.immunol.17.1.297.

[2] A. Bendelac, M.N. Rivera, S.H. Park, J.H. Roark, Mouse CD1-specific NK1 T cells: development, specificity, and function., Annu. Rev. Immunol. 15 (1997) 535–62. https://doi.org/10.1146/annurev.immunol.15.1.535.

[3] M. Brigl, M.B. Brenner, CD1: antigen presentation and T cell function., Annu. Rev. Immunol. 22 (2004) 817–90. https://doi.org/10.1146/annurev.immunol.22.012703.104608.

[4] Y. Kinjo, D. Wu, G. Kim, G.-W. Xing, M.A. Poles, D.D. Ho, M. Tsuji, K. Kawahara, C.-H. Wong, M. Kronenberg, Recognition of bacterial glycosphingolipids by natural killer T cells., Nature. 434 (2005) 520–5. https://doi.org/10.1038/nature03407.

[5] P.J. Brennan, T.-Y.Y. Cheng, D.G. Pellicci, G.F.M.M. Watts, N. Veerapen, D.C. Young, J. Rossjohn, G.S. Besra, D.I. Godfrey, M.B. Brenner, D.B. Moody, Structural determination of lipid antigens captured at the CD1d-T-cell receptor interface., Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 8348–8353. https://doi.org/10.1073/pnas.1705882114.

[6] M. Bonneville, R.L. O'Brien, W.K. Born, Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity., Nat. Rev. Immunol. 10 (2010) 467–78. https://doi.org/10.1038/nri2781.

[7] M. Koch, V.S. Stronge, D. Shepherd, S.D. Gadola, B. Mathew, G. Ritter, A.R. Fersht, G.S. Besra, R.R. Schmidt, E.Y. Jones, V. Cerundolo, The crystal structure of human CD1d with and without alpha-galactosylceramide., Nat. Immunol. 6 (2005) 819–826. https://doi.org/10.1038/ni1225.

[8] J.-J. Park, S.-J. Kang, A.D. De Silva, A.K. Stanic, G. Casorati, D.L. Hachey, P. Cresswell, S. Joyce, Lipid-protein interactions: biosynthetic assembly of CD1 with lipids in the endoplasmic reticulum is evolutionarily conserved., Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 1022–6. https://doi.org/10.1073/pnas.0307847100.

[9] S. Joyce, A.S. Woods, J.W. Yewdell, J.R. Bennink, A.D. De Silva, A. Boesteanu, S.P. Balk, R.J. Cotter, R.R. Brutkiewicz, Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol., Science. 279 (1998) 1541–4. https://doi.org/10.1126/science.279.5356.1541.

[10] Y. Kinjo, E. Tupin, D. Wu, M. Fujio, R. Garcia-Navarro, M.R.-E.-I. Benhnia, D.M. Zajonc, G. Ben-Menachem, G.D. Ainge, G.F. Painter, A. Khurana, K. Hoebe, S.M. Behar, B. Beutler, I.A. Wilson, M. Tsuji, T.J. Sellati, C.-H. Wong, M. Kronenberg, Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria., Nat. Immunol. 7 (2006) 978–86. https://doi.org/10.1038/ni1380.

[11] J.L. Amprey, J.S. Im, S.J. Turco, H.W. Murray, P.A. Illarionov, G.S. Besra, S.A. Porcelli, G.F. Späth, A subset of liver NK T cells is activated during Leishmania donovani infection by CD1d-bound lipophosphoglycan., J. Exp. Med. 200 (2004) 895–904. https://doi.org/10.1084/jem.20040704.

[12] M. Kronenberg, L. Gapin, The unconventional lifestyle of NKT cells., Nat. Rev. Immunol. 2 (2002) 557–68. https://doi.org/10.1038/nri854.

[13] C. Ma, M. Han, B. Heinrich, Q. Fu, Q. Zhang, M. Sandhu, D. Agdashian, M. Terabe, J.A. Berzofsky, V. Fako, T. Ritz, T. Longerich, C.M. Theriot, J.A. McCulloch, S. Roy, W. Yuan, V. Thovarai, S.K. Sen, M. Ruchirawat, F. Korangy, X.W. Wang, G. Trinchieri, T.F. Greten, Gut microbiome-mediated bile

acid metabolism regulates liver cancer via NKT cells., Science. 360 (2018) eaan5931. https://doi.org/10.1126/science.aan5931.

[14] S. Kojo, Y. Adachi, H. Keino, M. Taniguchi, T. Sumida, Dysfunction of T cell receptor AV24AJ18+, BV11+ double-negative regulatory natural killer T cells in autoimmune diseases., Arthritis Rheum. 44 (2001) 1127–38. https://doi.org/10.1002/1529-0131(200105)44:5<1127::AID-ANR194>3.0.CO;2-W.

[15] Y. Oishi, A. Sakamoto, K. Kurasawa, H. Nakajima, A. Nakao, N. Nakagawa, E. Tanabe, Y. Saito, I. Iwamoto, CD4-CD8- T cells bearing invariant Valpha24JalphaQ TCR alpha-chain are decreased in patients with atopic diseases., Clin. Exp. Immunol. 119 (2000) 404–11. https://doi.org/10.1046/j.1365-2249.2000.01157.x.

[16] M. Brigl, L. Bry, S.C. Kent, J.E. Gumperz, M.B. Brenner, Mechanism of CD1d-restricted natural killer T cell activation during microbial infection., Nat. Immunol. 4 (2003) 1230–7. https://doi.org/10.1038/ni1002.

[17] T. Kato, M. Kajikawa, K. Maenaka, E.Y. Park, Silkworm expression system as a platform technology in life science., Appl. Microbiol. Biotechnol. 85 (2010) 459–70. https://doi.org/10.1007/s00253-009-2267-2.

[18] X. Shi, D.L. Jarvis, Protein N-glycosylation in the baculovirus-insect cell system., Curr. Drug Targets. 8 (2007) 1116–25. https://doi.org/10.2174/138945007782151360.

[19] D.L. Jarvis, Developing baculovirus-insect cell expression systems for humanized recombinant glycoprotein production., Virology. 310 (2003) 1–7. https://doi.org/10.1016/s0042-6822(03)00120-x.

[20] D.G. Pellicci, O. Patel, L. Kjer-Nielsen, S.S. Pang, L.C. Sullivan, K. Kyparissoudis, A.G. Brooks, H.H. Reid, S. Gras, I.S. Lucet, R. Koh, M.J. Smyth, T. Mallevaey, J.L. Matsuda, L. Gapin, J. McCluskey, D.I. Godfrey, J. Rossjohn, Differential Recognition of CD1d-α-Galactosyl Ceramide by the Vβ8.2 and Vβ7 Semi-invariant NKT T Cell Receptors, Immunity. 31 (2009) 47–59. https://doi.org/10.1016/j.immuni.2009.04.018.

[21] T. Motohashi, T. Shimojima, T. Fukagawa, K. Maenaka, E.Y. Park, Efficient large-scale protein production of larvae and pupae of silkworm by Bombyx mori

nuclear polyhedrosis virus bacmid system., Biochem. Biophys. Res. Commun. 326 (2005) 564–9. https://doi.org/10.1016/j.bbrc.2004.11.060.

[22] K. Sasaki, M. Kajikawa, K. Kuroki, T. Motohashi, T. Shimojima, E.Y. Park, S. Kondo, H. Yagi, K. Kato, K. Maenaka, Silkworm expression and sugar profiling of human immune cell surface receptor, KIR2DL1., Biochem. Biophys. Res. Commun. 387 (2009) 575–80. https://doi.org/10.1016/j.bbrc.2009.07.065.

[23] M. Kajikawa, K. Sasaki, Y. Wakimoto, M. Toyooka, T. Motohashi, T. Shimojima, S. Takeda, E.Y. Park, K. Maenaka, Efficient silkworm expression of human GPCR (nociceptin receptor) by a Bombyx mori bacmid DNA system., Biochem. Biophys. Res. Commun. 385 (2009) 375–9. https://doi.org/10.1016/j.bbrc.2009.05.063.

[24] N.A. Borg, K.S. Wun, L. Kjer-Nielsen, M.C.J. Wilce, D.G. Pellicci, R. Koh, G.S. Besra, M. Bharadwaj, D.I. Godfrey, J. McCluskey, J. Rossjohn, CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor., Nature. 448 (2007) 44–9. https://doi.org/10.1038/nature05907.

[25] E. Mottez, C. Jaulin, F. Godeau, J. Choppin, J.-P. Levy, P. Kourilsky, A single-chain murine class I major transplantation antigen, Eur. J. Immunol. 21 (1991) 467–471. https://doi.org/10.1002/eji.1830210232.

[26] T. Primeau, N.B. Myers, Y.Y.L. Yu, L. Lybarger, X. Wang, S.M. Truscott, T.H. Hansen, J.M. Connolly, Applications of major histocompatibility complex class I molecules expressed as single chains., Immunol. Res. 32 (2005) 109–21. https://doi.org/10.1385/IR:32:1-3:109.

[27] Y.Y.L. Yu, N. Netuschil, L. Lybarger, J.M. Connolly, T.H. Hansen, Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells., J. Immunol. 168 (2002) 3145–9. https://doi.org/10.4049/jimmunol.168.7.3145.

[28] D. Li, N. Chen, A.J. McMichael, G.R. Screaton, X.-N.N. Xu, Generation and characterisation of CD1d tetramer produced by a lentiviral expression system., J. Immunol. Methods. 330 (2008) 57–63. https://doi.org/10.1016/j.jim.2007.10.018.

[29] D.M. Zajonc, C. Cantu, J. Mattner, D. Zhou, P.B. Savage, A. Bendelac, I.A. Wilson, L. Teyton, Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor., Nat. Immunol. 6 (2005) 810–8.

https://doi.org/10.1038/ni1224.

[30] E. Kurimoto, K. Kuroki, Y. Yamaguchi, M. Yagi-Utsumi, T. Igaki, T. Iguchi, K. Maenaka, K. Kato, Structural and functional mosaic nature of MHC class I molecules in their peptide-free form., Mol. Immunol. 55 (2013) 393–9. https://doi.org/10.1016/j.molimm.2013.03.014.

[31] E. Honjo, Y. Shoyama, T. Tamada, H. Shigematsu, T. Hatanaka, S. Kanaji, K. Arima, Y. Ito, K. Izuhara, R. Kuroki, Expression of the extracellular region of the human interleukin-4 receptor alpha chain and interleukin-13 receptor alpha1 chain by a silkworm-baculovirus system., Protein Expr. Purif. 60 (2008) 25–30. https://doi.org/10.1016/j.pep.2008.03.020.

[32] T. Dojima, T. Nishina, T. Kato, T. Uno, H. Yagi, K. Kato, H. Ueda, E.Y. Park, Improved secretion of molecular chaperone-assisted human IgG in silkworm, and no alterations in their N-linked glycan structures., Biotechnol. Prog. 26 (2009) 232–8. https://doi.org/10.1002/btpr.313.

Figs

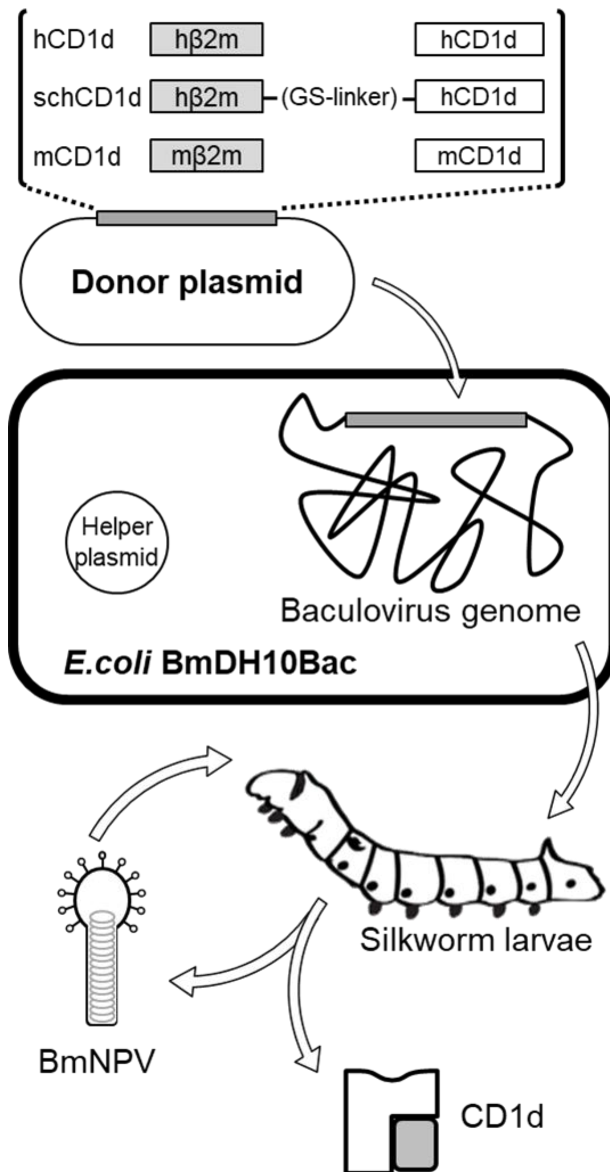


Fig. 1. The construction of CD1d proteins and the diagram of the silkworm-baculovirus expression system. The CD1d gene was incorporated into the BmNPV bacmid. This recombinant BmNPV bacmid extracted from *E. coli* injected directly into 5th instar silkworm larvae. The CD1d protein and the recombinant BmNPV were harvested after 6 days post-injection. This BmNPV was injected into silkworm larvae and the CD1d protein was harvested.

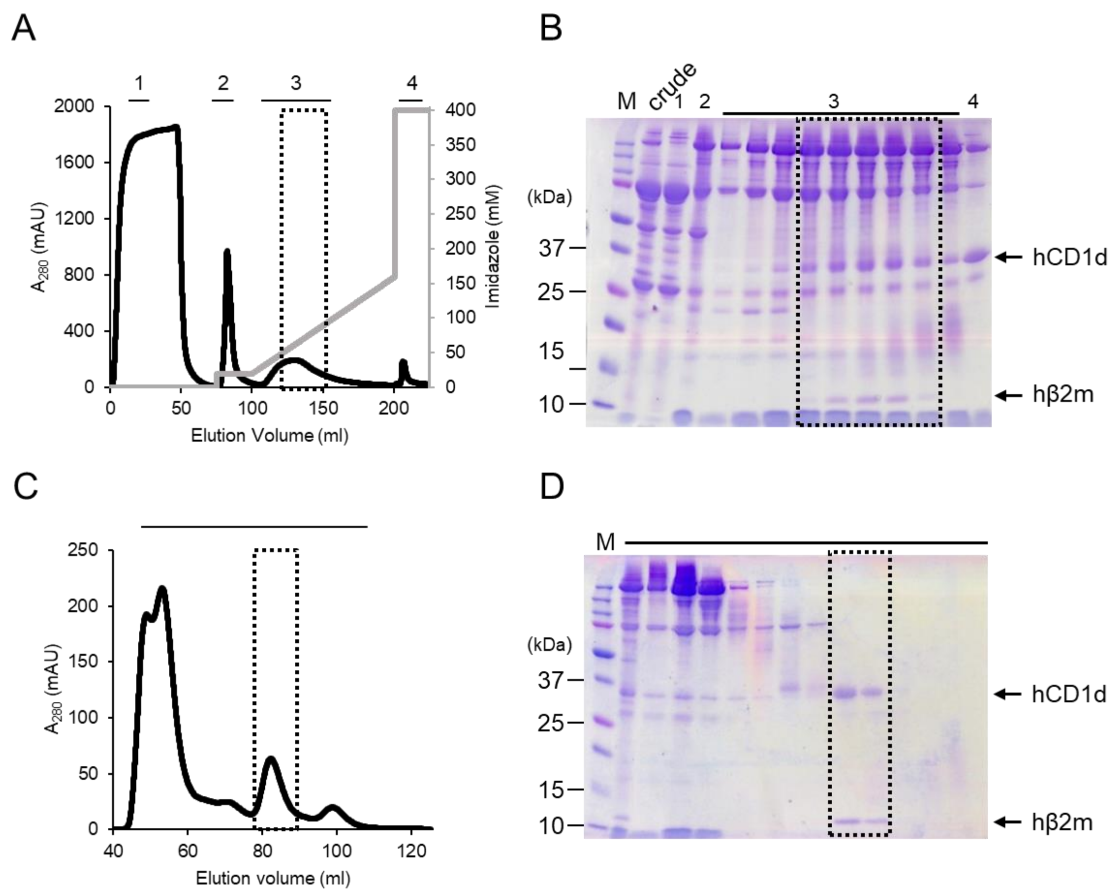


Fig. 2. The purification of hCD1d. (A) The chromatogram of Ni-affinity chromatography. (B) The result of SDS-PAGE analyzing each fraction of the Ni-affinity chromatography. The dashed line shows the presence of CD1d-β2m complex. M: protein molecular weight markers; crude: crude CD1d concentrated by ammonium sulfate fractionation; Lane 1: flowthrough fraction; Lane 2: wash fraction after sample loading; Range 3: elution fractions; Lane 4: wash fraction after elution. (C) The chromatogram of size exclusion chromatography. (D) The result of SDS-PAGE analyzing each fraction of the size exclusion chromatography. The dashed line shows the presence of CD1d- CD1d-β2m complex. M: protein molecular weight markers.

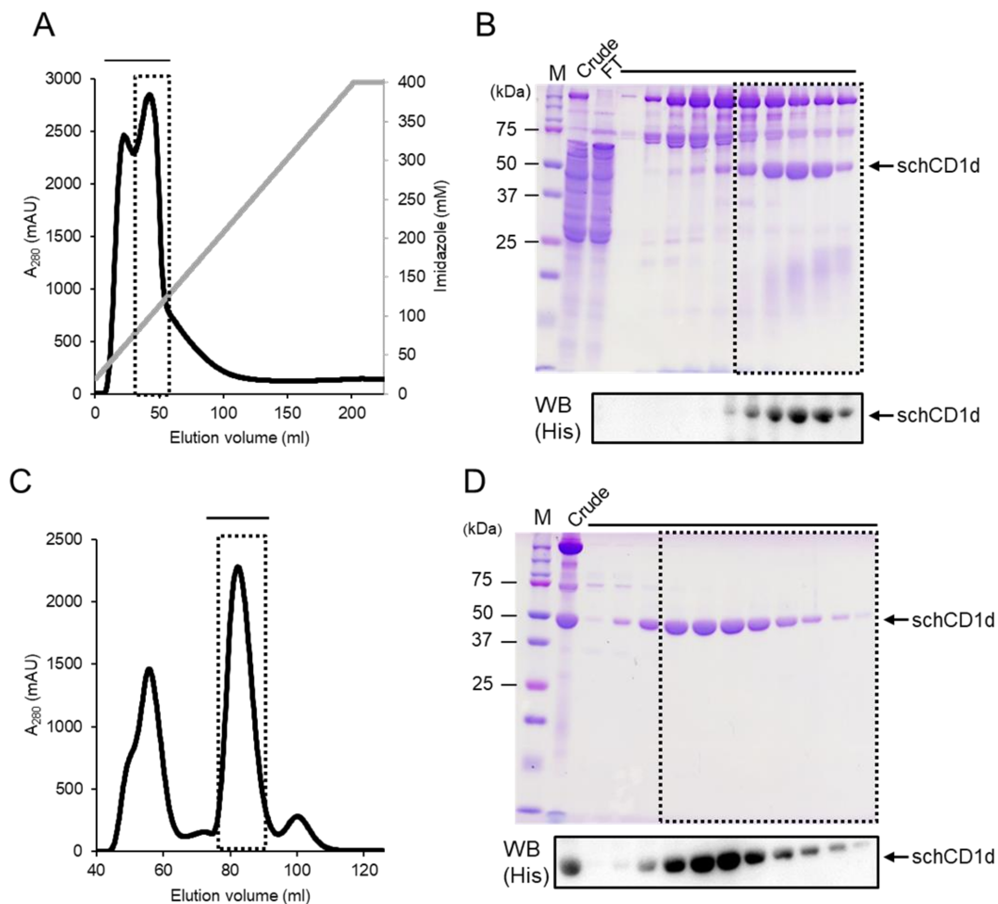


Fig. 3. The purification of schCD1d. (A) The chromatogram of Ni-affinity chromatography. (B) The result of SDS-PAGE (upper panel) and western blotting (lower panel) analyzing each fraction of the Ni-affinity chromatography. The dashed line shows the presence of schCD1d. M: protein molecular weight markers; crude: crude CD1d concentrated by ammonium sulfate fractionation; FT: flowthrough fraction. (C) The chromatogram of size exclusion chromatography. (D) The result of SDS-PAGE (upper panel) and western blotting (lower panel) analyzing each fraction of the size exclusion chromatography. The dashed line shows the presence of schCD1d. M: protein molecular weight markers; crude: collected fractions after Ni-affinity chromatography.

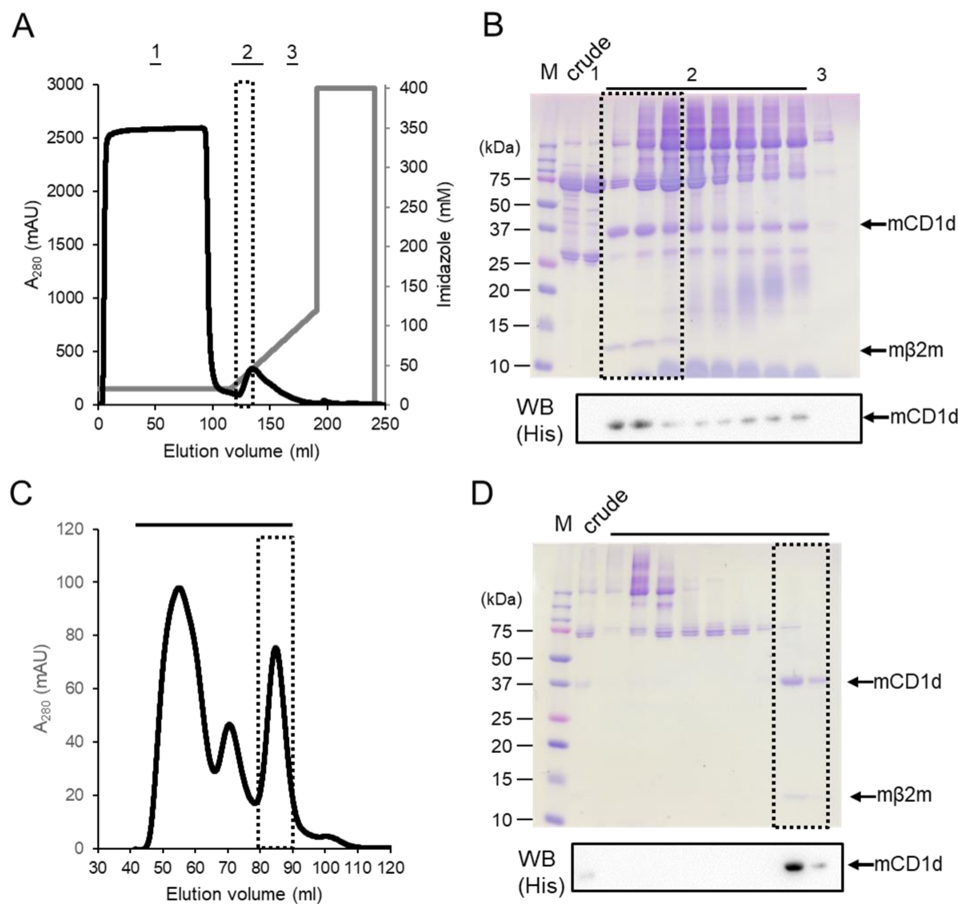


Fig. 4. The purification of mCD1d. (A) The chromatogram of Ni-affinity chromatography. (B) The result of SDS-PAGE (upper panel) and western blotting (lower panel) analyzing each fraction of the Ni-affinity chromatography. The dashed line shows the presence of mCD1d. M: protein molecular weight markers; crude: crude CD1d concentrated by ammonium sulfate fractionation; Lane 1: flowthrough fraction; Range 2 and Lane 3: elution fractions. (C) The chromatogram of size exclusion chromatography. (D) The result of SDS-PAGE (upper panel) and western blotting (lower panel) analyzing each fraction of the size exclusion chromatography. The dashed line shows the presence of mCD1d. M: protein molecular weight markers; crude: collected fractions after Ni-affinity chromatography.

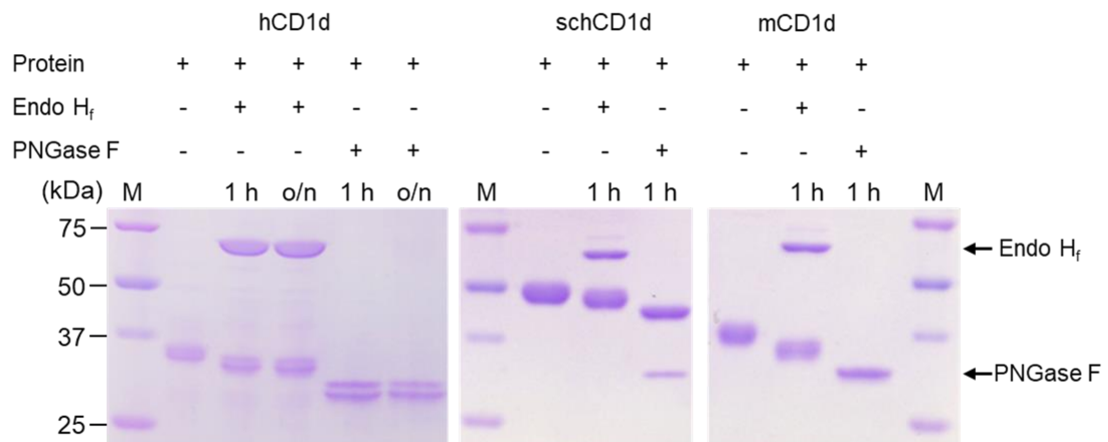
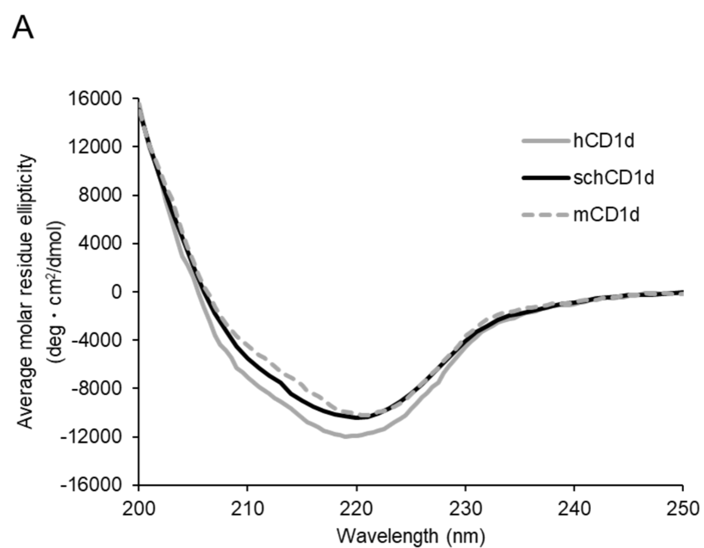
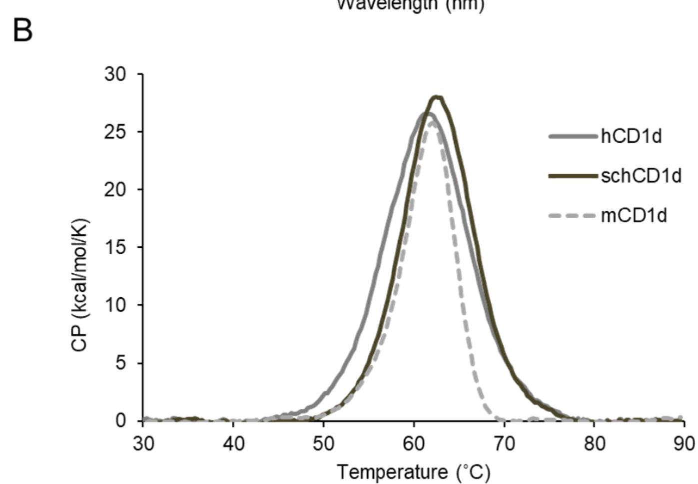


Fig. 5. SDS-PAGE images analyzing the CD1d digested by endoglycosidases. End H_f digestion splits hCD1d (left) into two bands, and hCD1d became one band in the lower position due to digestion of PNGase F. The upper band of PNGase F^+ hCD1d is PNGase F. The schCD1d (middle) and the mCD1d (right) were digested similarly to hCD1d. The band of mCD1d digested by PNGase F overlaps with the band of PNGase F.

A

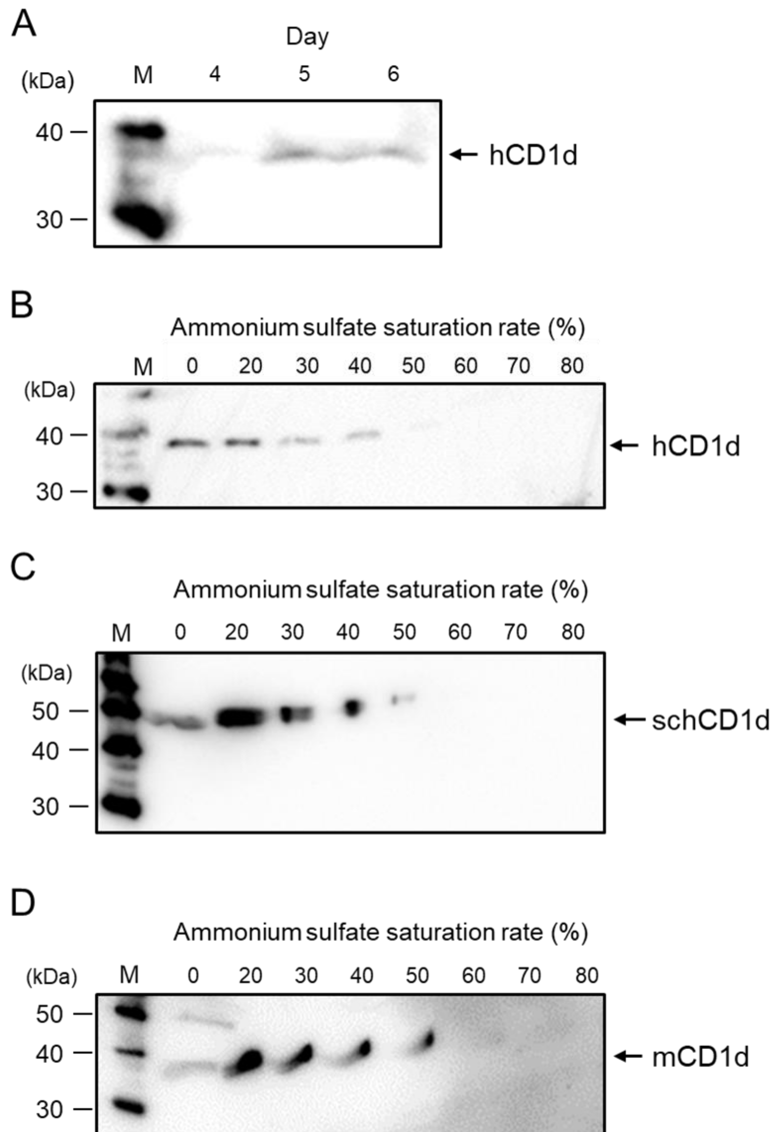


B

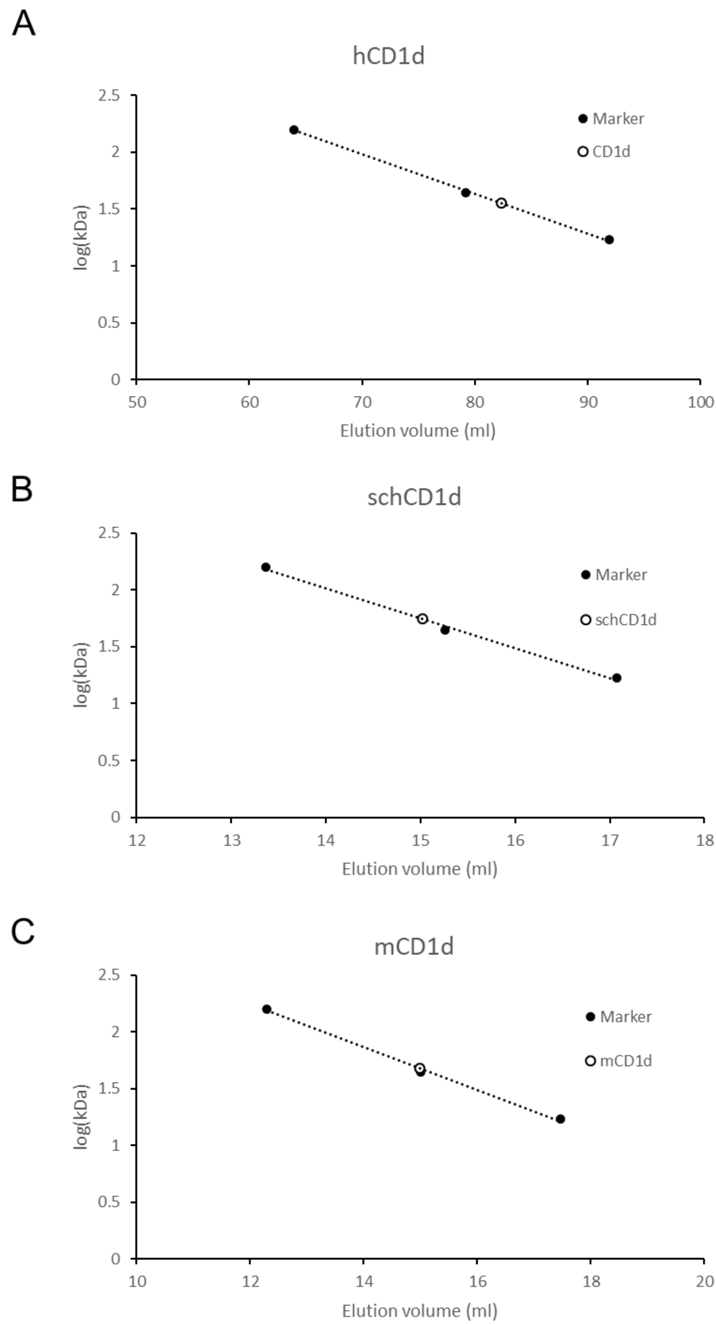


	T_m (℃)
hCD1d	61.5
schCD1d	62.7
mCD1d	61.7

Fig. 6. Characterization of CD1d. (A) CD spectrum of CD1d proteins. (B) DSC curves and Tm value of CD1d proteins.



Supplementary Fig. 1. (A) Western blotting analysis using the hemolymph after injection of BmNPV bacmid. (B)-(D) Western blotting analysis using supernatant of ammonium sulfate precipitation.



Supplementary Fig. 2. Standard curve for size exclusion chromatography. The filled circles indicate the positions of the protein standard. The open circles indicate the positions of peak of (A) hCD1d, (B) schCD1d and (C) mCD1d, respectively.