Insect Biochemistry and Molecular Biology 43 (2013) 612–625

Contents lists available at SciVerse ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Gloverins of the silkworm *Bombyx mori*: Structural and binding properties and activities

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ARTICLE INFO

Article history:
Received 15 January 2013
Received in revised form
21 March 2013
Accepted 26 March 2013

Keywords:
Gloverin
Lipopolysaccharide
Circular dichroism
Antibacterial
Random coil
α-helix
Bombyx mori

ABSTRACT

Gloverins are basic, glycine-rich and heat-stable antibacterial proteins (~14- kDa) in lepidopteran insects with activity against *Escherichia coli*, Gram-positive bacteria, fungi and a virus. *Hyalophora gloveri* gloverin adopts a random coil structure in aqueous solution but has α-helical structure in membrane-like environment, and it may interact with the lipid A moiety of lipopolysaccharide (LPS). *Manduca sexta* gloverin binds to the O-specific antigen and outer core carbohydrate of LPS. In the silkworm *Bombyx mori*, there are four gloverins with slightly acidic to neutral isoelectric points. In this study, we investigate structural and binding properties and activities of *B. mori* gloverins (*Bm*Glvs), as well as correlations between structure, binding property and activity. Recombinant *Bm*Glv1-4 were expressed in bacteria and purified. Circular dichroism (CD) spectra showed that all four *Bm*Glvs mainly adopted random coli structure (>50%) in aqueous solution in regardless of pH, but contained α-helical structure in the presence of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), smooth and rough mutants (Ra, Rc and Re) of LPS and lipid A. Plate ELISA assay showed that *Bm*Glvs at pH 5.0 bound to rough mutants of LPS and lipid A but not to smooth LPS. Antibacterial activity assay showed that positively charged *Bm*Glvs (at pH 5.0) were active against *E. coli* mutant strains containing rough LPS but inactive against *E. coli* with smooth LPS. Our results suggest that binding to rough LPS is the prerequisite for the activity of *Bm*Glvs against *E. coli*.

1. Introduction

Insects have the largest numbers and species on earth, and they combat a variety of pathogens mainly relying on sophisticated innate immune system. Insect defense system consists of three major parts: structural barriers, cellular and humoral immune responses (Lemaitre and Hoffmann, 2007). Structural barriers, the first protective lines, refer to cuticle, midgut epithelium and trachea. Cellular immune responses, including phagocytosis, nodulation and encapsulation, are mediated by several types of hemocytes (Lavine and Strand, 2002; Sideri et al., 2007). Humoral immune responses include melanization of hemolymph and secretion of antimicrobial peptides (AMPs) (Hoffmann, 1995). AMPs, the major and best known immune effectors induced by infection, are synthesized by fat body, hemocytes and other tissues, and regulated by the Toll and immune deficiency (IMD) pathways (Bulet et al., 1999; Lemaitre and Hoffmann, 2007).

At least 150 insect AMPs have been purified or identified. Most insect AMPs are small and cationic, and they show activities against bacteria and/or fungi (Hoffmann, 1995; Bulet and Stocklin, 2005). Based on the sequences, structures and activities, insect AMPs can be classified into four families, the α-helical peptides (e.g., cecropin, moricin and sarcotoxin), cysteine-rich peptides (e.g., insect defensin, drosomycin and heliomicin), proline-rich peptides (e.g., apidaecin, drosocin and lebocin), and glycine-rich peptides (e.g., attacin and gloverin) (Laszlo-Otvos, 2000; Bulet and Stocklin, 2005). Glycine-rich peptides with molecular masses of more than 10 kDa have become a large family of insect AMPs, including attacin, sarcotoxin II, gloverin, hymenoptaecin, coleoptericin, hemipterecin and tenecin 4 (Hultmark et al., 1983; Ando et al., 1987; Axen

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http://dx.doi.org/10.1016/j.ibmb.2013.03.013

et al., 1997; Casteels et al., 1993; Bulet et al., 1991; Cociancich et al., 1994; Chae et al., 2012).

So far, small cationic AMPs (~4 kDa) have been the focus of structural and activity study. Cysteine-rich peptides, which are cyclic by formation of disulfide bonds, can form stable structures in aqueous solution. However, linear AMPs do not form stable structures in aqueous solution but can change to stable structures in membrane mimic environment (Nguyen et al., 2008; Haney and Vogel, 2009). Cysteine-rich AMPs, such as insect defensin A, drosomycin, termicin and heliomicin, contain "cysteine stabilized αβ motif" (CSαβ) structure with antiparallel β-sheet connected to a single α-helix by two disulfide bridges (Cornet et al., 1995; Landon et al., 1997; Silva et al., 2003; Lamberty et al., 2001). But nearly all the structures of α-helical linear AMPs are obtained by using micelle suspensions or in the presence of organic solvents. For example, *Hyalophora cecropia* cecropin A exists as random coli in aqueous solution but forms an amphipathic helical structure in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/water solution (Holak et al., 1988). The α-helical structure of moricin is also obtained in methanol and in solution containing 2,2,2-trifluoroethanol (TFE) or sodium dodecylsulphate (SDS) (Dai et al., 2008).

Since the first purification and characterization of gloverin from *Hyalophora gloveri* pupal hemolymph (Axen et al., 1997), a gloverin has been isolated from *Helicoverpa armigera* (Mackintosh et al., 1998), and two gloverins have been detected in hemolymph of septic injured *Diatraea saccharialis* larvae (Silva et al., 2010). Gloverin genes have also been identified in *Antheraea mylitta* (Gandhe-Archana et al., 2006), *Galleria mellonella* (Seitz et al., 2003; Brown et al., 2009), *Manduca sexta* (Abdel-Latief and Hilker, 2008; Xu et al., 2012; Zhu et al., 2003), *Plutella xylostella* (Eum et al., 2007; Etebari et al., 2011), *Spodoptera exigua* (Hwang and Kim, 2011), and *Trichoplusia ni* (Lundstrom et al., 2002). In the silkworm *Bombyx mori*, four gloverin genes (*Bmglv*1-4) have been identified, and *Bmglv*2-4 genes are derived from duplication of *Bmglv*1 (Cheng et al., 2006; Kaneko et al., 2007; Kawaoka et al., 2008; Mrinal and Nagaraju, 2008). Among the gloverins with known activities, *H. gloveri* gloverin is active against *Escherichia coli* D21f2 and D21 mutant strains with rough LPS (Axen et al., 1997), *H. armigera* gloverin is active against *E. coli* strains with smooth LPS and D22 strain that is defective in lipid A (Mackintosh et al., 1998), *T. ni* gloverin 1 and 2 have activity against *E. coli* D21f2 and D22 strains and a virus (Lundstrom et al., 2002; Moreno-Habel et al., 2012). However, *S. exigua* gloverin is active against a Gram-positive bacterium (*Flavobacterium* sp.) but inactive against *E. coli* strain with smooth LPS (Hwang and Kim, 2011), *M. sexta* gloverin shows activity against a Gram-positive *Bacillus cereus* and two fungi (*Saccharomyces cerevisiae* and *Cryptococcus neoformans*) but inactive against *E. coli* strain with smooth LPS (Xu et al., 2012).

The majority of gloverins is basic or highly basic (*p*I ~8.3 for *H. gloveri* gloverin, *T. ni* gloverin 1 and *S. exigua* gloverin, *p*I > 9.0 for most other gloverins) and heat-stable with high content (>18%) of glycine residues (Xu et al., 2012). *H. gloveri* gloverin (*Hg*Glv, *p*I ~8.3) can inhibit the growth of *E. coli* by inhibiting synthesis of bacterial outer membrane proteins and increasing permeability of the membrane (Axen et al., 1997). Basic gloverins may interact with lipopolysaccharide (LPS) via charge–charge interaction with negatively charged lipid A (Axen et al., 1997). But direct binding of gloverin to microbial components including LPS has only been reported for *M. sexta* gloverin (*Ms*Glv, *p*I ~9.3) (Xu et al., 2012). Recombinant *Ms*Glv can bind to the O-specific antigen and outer core carbohydrate moieties of LPS, Gram-positive lipoteichoic acid (LTA) and peptidoglycan (PG), and laminarin, but does not bind to lipid A (Xu et al., 2012). Known gloverins with acidic or neutral *p*I include *Heliothis virescens* gloverin (*p*I ~7.2) (Genbank accession number: ACR78446), *A. mylitta* gloverin 2 (*p*I ~6.8) (Genbank accession number: ABG72700), and four *B. mori* gloverins (*Bm*Glvs) (*p*I ~5.5, 7.0, 6.3 and 7.0 for *Bm*Glv1-4, respectively) (Kawaoka et al., 2008). Recombinant *Bm*Glvs show activity against *E. coli* strains with smooth LPS (Kawaoka et al., 2008; Mrinal and Nagaraju, 2008). However, it is not clear whether *Bm*Glvs can interact with LPS and whether they also adopt random coil structures in aqueous solution and undergo conformational transitions in the hydrophobic environment. In this study, we investigate structural transitions of *Bm*Glvs in the hydrophobic environment (organic solvent, detergent micelles and LPS), binding properties of *Bm*Glvs to LPS and other microbial cell wall components, and antibacterial activities of *Bm*Glvs against *E. coli* strains with smooth and rough mutant forms of LPS.

2. Materials and methods

2.1. Microorganisms and microbial components

E. coli DH5α (TIANGEN, China) and *E. coli* Rosetta™ (DE3) (Transgen, China) strains were used to clone and express recombinant *Bm*Glvs. *E. coli* (ATCC 25922), *Pichia pastoris*, *Serratia marcescens* and *Bacillus thuringiensis* were from American Type Culture Collection (ATCC). *Staphylococcus aureus* and *B. cereus* were kindly provided by Professor Brian Geisbrecht, *S. cerevisiae* (BY4741) and *C. neoformans* (alpha) were provided by Professor Alexander Idnurm, *Bacillus subtilis* was provided by Professor Michael O'Connor, School of Biological Sciences at University of Missouri – Kansas City. *E. coli* D21, D21e7, D21f1 and D21f2 strains with rough mutants of LPS were purchased from *E. coli* Genetic Resources at Yale CGSC, The coli Genetic stock center (USA).

Smooth LPS from *Salmonella enterica*, *S. marcescens*, *E. coli* 055:B5, *E. coli* 026:B6 and *E. coli* 0111:B4, rough mutants of LPS from *E. coli* EH100 (Ra mutant), *E. coli* J5 (Rc mutant), *E. coli* F583 (Rd mutant) and *S. enterica* serotype minnesota Re 595 (Re mutant), as well as lipid A monophosphoryl from *E. coli* F583 (Rd mutant), laminarin, mannan, and zymosan were from Sigma–Aldrich (MO, USA) and used for binding assay. TLRgrade LPS and PG from *E. coli* K12 (LPS-K12 and PG-K12), TLRgrade peptidoglycan (PG) and lipoteichoic acid (LTA) from *B. subtilis* (LTA-BS and PG-BS) and *S. aureus* (LTA-SA and PG-SA) were from Invivogen (CA, USA) and also used for binding assay. LPS from *E. coli* serotype 055:B5 (smooth LPS), *E. coli* serotype EH100 (Ra), *E. coli* serotype J5 (Rc) and *E. coli* serotype R515 (Re), and monophosphoryl lipid A from *E. coli* serotype R515 were purchased from Alexis (Alexis, Switzerland) and used for circular dichroism (CD) experiments.

2.2. Construction of expression vectors for recombinant B. mori gloverins

Total RNAs were isolated from the fat body of day-3 fifth instar *B. mori* larvae collected at 24 h after injection of *E. coli* (ATCC 25922) (1×10^5 cells per larva) using TRIzol Reagent (Invitrogen), and the first strand cDNA was synthesized using M-MLV Reverse Transcriptase (TOYOBO, Japan). RT-PCR was performed to obtain cDNA sequences encoding *B. mori* mature gloverins using the following primer pairs: 5′-CAT GCC ATG GAT ATT CAC GAC TTT GTC AC-3′ and 5′-CGC CTC GAG CCA CTC GTG AGT AAT CTG-3′ (for mature *Bm*Glv1, residues 44–178), 5′-CAT GCC ATG GAC GTC ACT TGG GAC AAA CAA-3′ and 5′-CAG CTC GAG CCA ATC ATG GCG GAT CTC TG-3′ (for mature *Bm*Glv2, residues 43–173), 5′-CGA TCC ATG GAC GTC ACG TGG GAC ACG-3′ and 5′-CCG CTC GAG CCA CTC ATG CCG GAT CTC-3′ (for mature *Bm*Glv3, residues 43–173), 5′-CAT GCC ATG GAC GTC ACC TGG GAC AAA CAA G-3′ and 5′-CCG CTC GAG CCA ATC ATG GCG GAA CTC T-3′ (for mature *Bm*Glv4, residues 41–171). PCR products were purified using the EZNA cycle-pure kit (Omega, USA) and