Identification and Cloning of a Glucan- and Lipopolysaccharide-binding Protein from *Eisenia foetida* Earthworm Involved in the Activation of Prophenoloxidase Cascade*

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Coelomic fluid of *Eisenia foetida* earthworms contains a 42-kDa protein named coelomic cytolytic factor 1 (CCF-1) that was described previously to be involved in cytolytic, opsonizing, and hemolytic properties of the coelomic fluid. Cloning and sequencing of CCF-1 reveal significant homology with the putative catalytic region of β-1,3- and β-1,3–1,4-glucanases. CCF-1 also displays homology with coagulation factor G from *Limulus polyphemus* and with Gram-negative bacteria-binding protein of *Bombyx mori* silkworm, two proteins involved in invertebrate defense mechanisms. We show that CCF-1 efficiently binds both β-1,3-glucan and lipopolysaccharide. Moreover, CCF-1 participates in the activation of prophenoloxidase cascade via recognition of yeast and Gram-negative bacteria cell wall components. These results suggest that the 42-kDa CCF-1 protein of *E. foetida* coelomic fluid likely plays a role in the protection of earthworms against microbes.

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The prophenoloxidase (pro-PO) activating system represents an important defense mechanism in a large variety of invertebrates (reviewed in Refs. 1 and 2). This system is based on the recognition of bacterial antigens such as lipopolysaccharide (LPS) or peptidoglycan and 1,3-glucan present as major components of the cell wall of yeast and fungi (3, 4). Generally, upon the recognition of such saccharides proteases cleave by limited proteolysis inactive pro-PO to its active state, phenoloxidase (PO). The active enzyme catalyzes the o-hydroxylation of monophenols as well as the oxidation of diphenols to quinones that are subsequently polymerized nonenzymatically to melanin. Melanin and its precursors involved in the pro-PO-activating system have cytotoxic and antimicrobial properties and participate in a wide range of other biological activities including phagocytosis/opsonization, encapsulation/nodule formation, degranulation, and wound healing (5–9).

The pro-PO-activating system has been detected both in protostomian and deuterostomian species. Although pro-PO-activating system is well documented in arthropods, data in other protostomian groups are more scarce. In annelids, melamination reactions and formation of “brown bodies” or nodules have been described in polychaetes and oligochaetes (10–14). However, biochemical detection of PO activity was so far restricted to a few species with rather controversial results. Whereas Smith and Söderhäll (15) failed to detect pro-PO system in the polychaete *Aphrodite acetacea* and * Arenicola marina*, Fischer (16), Valemois et al. (17), and Porchet-Henrè and Vernet (13) have documented PO activity in *Lambri- cus terrestris*, *Eisenia fetida andrei*, and *Nereis diversicolor*, respectively. More recently using L-DOPA as substrate, a 38-kDa protein responsible for PO activity was identified in the coelomic fluid of *E. foetida andrei* (18). A report showing that the oxidative activity of the coelomic fluid of earthworms toward L-DOPA in vitro is not affected by trypsin but completely blocked by subtilisin reflects the importance of a correct proteolytic digestion as an initial step for inactive pro-PO activation (17).

Since the factor that recognizes microbial saccharides and triggers the pro-PO system has not yet been described in annelids (1, 2), investigations were initiated to identify such molecules in the coelomic fluid of *E. foetida foetida*. Surprisingly, we found that a previously described 42-kDa protein named CCF-1 (coelomic cytolytic factor 1; see Ref. 19) binds specifically β-1,3-glucan and LPS from the smooth (S) chemotype. Cloning and sequence analysis show that CCF-1 displays significant amino acid homology with β-1,3- and β-1,3–1,4-glucanases and furthermore shares similarity with clotting factor G from horseshoe crab and a Gram-negative binding protein from silkworm. Finally, we report that CCF-1 participates in the pro-PO cascade in the coelomic fluid of *E. foetida*.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cell-free coelomic fluid was isolated from adult specimens of *E. foetida foetida* (Oligochaeta; Annelida) in the absence of serine protease inhibitor as described (19). Gram-negative *Salmonella typhimurium*
coelomic fluid with TN buffer (S), *Salmonella minnesota* (S), *E. coli* (Ra), and *S. typhimurium* (S, Rd) were purchased from Sigma. All LPS fractions were under reducing conditions. C, elution of S-LPS-bound material after preincubating coelomic fluid with TN buffer (a), laminarin (b), S-LPS from *S. typhimurium* (c), S-LPS from *E. coli* 055:B5 (d), cellobiose (e), or Re-LPS (f) (samples under reducing conditions). D, Western blot of curdlan- (a) or S-LPS-bound (b) material from coelomic fluid visualized by anti-CCF-1 mAb (samples under reducing conditions).

*Fig. 1. Detection and isolation of glucan- and LPS-binding protein in the coelomic fluid of *E. foetida*. A, CF, total coelomic fluid proteins (silver staining); a and b, nonreduced and reduced glucan-binding protein in the coelomic fluid identified using biotinylated laminarin (ligand blot); c and d, nonreduced and reduced curdlan-bound material eluted from coelomic fluid (silver staining); e and f, nonreduced and reduced *S. typhimurium* LT2 S-LPS-bound material eluted from coelomic fluid (silver staining). B, elution of curdlan-bound material after preincubating coelomic fluid with TN buffer (a), laminarin (b), S-LPS from *S. typhimurium* (c), S-LPS from *E. coli* 055:B5 (d), cellobiose (e), or Re-LPS (f) (samples under reducing conditions). C, elution of S-LPS-bound material after preincubating coelomic fluid with TN buffer (a), laminarin (b), S-LPS from *S. typhimurium* (c), S-LPS from *E. coli* 055:B5 (d), cellobiose (e), or Re-LPS (f) (samples under reducing conditions). D, Western blot of curdlan- (a) or S-LPS-bound (b) material from coelomic fluid visualized by anti-CCF-1 mAb (samples under reducing conditions).*
Expression of Recombinant CCF-1 in E. coli

Construction of pIGRHISA-CCF-1 Vector—The cDNA sequence encoding for mature CCF-1 (mCCF-1) was amplified by PCR using PWO polymerase (Boehringer Mannheim) and the pBluescript phagemid as template. The primers were designed so that after PCR, the mCCF-1 cDNA contained BamHI/SalI sites at the 5’ end (GGGATCCGATCT-TCACCGACTGGATGATCAATATC) and a SalI site at the 3’ end (CCGCCTCGAGGCAGCAATCTAATGCAATCTA). Hence, after cutting the PCR product with SalI and blunting the sticky ends, the first codon of the mCCF-1 was blunt-end available for ligation. The BamHI-SalI fragment was cloned into pBluescript (pBSmCCF-1) and checked by sequencing. A SalI-blunted SalI fragment containing the mCCF-1 cDNA from pBSmCCF-1 was cloned into pIGHRSA vector (provided by Innogenetics, Zwijnaarde, Belgium). In this vector an amino-terminal His tag and an enterokinase cleavage site precede the mCCF-1 cDNA sequence. After transformation in the E. coli strain MC1061, the clones were ready for induction.

Induction and Purification of Recombinant CCF-1 Protein (rCCF-1)—Since the expression of pIGHRSA-CCF-1 is under the control of the PL promoter, cultures were grown at 32 °C and induced at 42 °C at an Aabs of 0.7. One liter of induced bacterial culture was resuspended in PBS and sonicated. After centrifugation the pellet was solubilized in 50 ml of a urea solution (8 M urea in TN buffer), applied on 2.5 ml of Ni-NTA agarose resin (Qiagen), and renatured by a linear decreasing gradient of urea solution while rCCF-1 was bound to the column. Three successive elutions were performed by imidazole (300 mM imidazole in TN buffer). Imidazole was removed by extensive dialysis against TN buffer.

Laminarin and LPS Binding to Immobilized rCCF-1

Microtitre plates were coated with rCCF-1 (100 μg/ml of well) of 10 μg/ml in TN buffer, overnight, 4 °C. Free sites were blocked with 1% BSA in TN buffer (200 μg/ml well, 3 h, 37 °C). After washings (TN buffer) different doses of FITC-labeled laminarin or FITC-labeled S-LPS were added (in 100 μl of TN buffer, 0.1% BSA). After 3 h at 37 °C, plates were washed; 100 μl well TN buffer were added, and the bound fluorescence was measured using a fluorescence multi-well plate reader CytoFluor II (PerSeptive Biosystems; excitation/emission 485 ± 20/530 ± 30 nm). Mean bound fluorescence ± S.D. of triplicates was then analyzed.

Control experiments were performed on BSA-coated plates. For inhibition experiments, rCCF-1-coated plates were incubated with different doses of inhibitors (in 100 μl TN buffer, 0.1% BSA, 3 h, 37 °C). After washings, FITC-labeled laminarin or FITC-labeled S-LPS was added (in 100 μl TN buffer, 0.1% BSA, 3 h, 37 °C), and CCF-1-bound material was quantified as described above. The dose of inhibitor reducing by 50% the binding of 50 μg/ml laminarin or 10 μg/ml LPS was estimated (ED50).

Prophenoloxidase-activating System

The level of the pro-PO system activation was assessed according to Valemois et al. (17). Briefly, in a total volume of 100 μl, 10 μl of coelomic fluid (with or without 1 mM Pefabloc (serine protease inhibitor, Boehringer Mannheim)), 80 μl of Tris, pH 8, containing 50 mM Ca2+, and 10 mM DOPA (3,4-dihydroxyphenyl-L)-alanine; Fluka; final concentration 1.5 mM) were incubated at room temperature for 6 h in the absence or presence of different doses of lyophilized microorganisms, saxocharides, or LPS fractions. The oxidation of L-DOPA was measured at 492 nm and expressed as the difference between the values without and with Pefabloc. To confirm the role of glucan- or LPS-binding protein in pro-PO activation, the coelomic fluid was incubated with anti-CCF-1 at 4 °C, centrifuged, and the depleted coelomic fluid was used in L-DOPA oxidation test as described above. To reconstitute the pro-PO activating cascade rCCF-1 (0.5–2 μg/ml) was added to CCF-1-depleted coelomic fluid before testing L-DOPA oxidation.

RESULTS

Detection of Glucan-binding Protein in the Coelomic Fluid of E. fetida—A putative β-1,3-glucan-binding protein was detected in the coelomic fluid of E. fetida transferred onto nitrocellulose after SDS-polyacrylamide gel electrophoresis using soluble biotin-labeled laminarin (β-1,3-glucan). Out of all coelomic fluid proteins, laminarin reacts under reducing and non-reducing conditions with only one band with apparent molecular mass (M6000 of 0.7). One liter of induced bacterial culture was resuspended in PBS and sonicated. After centrifugation the pellet was solubilized in 50 ml of a urea solution (8 M urea in TN buffer), applied on 2.5 ml of Ni-NTA agarose resin (Qiagen), and renatured by a linear decreasing gradient of urea solution while rCCF-1 was bound to the column. Three successive elutions were performed by imidazole (300 mM imidazole in TN buffer). Imidazole was removed by extensive dialysis against TN buffer.

Amino acid residues in boldface indicate the TGA stop codon.
Isolation of Glucan- and LPS-binding Protein from Earthworm Coelomic Fluid—To isolate putative glucan-binding protein(s) coelomic fluid of *E. foetida* was incubated with curdlan particles (β-1,3-glucan). Glucan-bound material was eluted with electrophoresis SDS sample buffer, subjected to electrophoresis, and silver-stained (Fig. 1A). Bound material consists of a single component with an apparent molecular mass of 42 kDa. By using LPS from *S. typhimurium* LT2 (S) which is insoluble at neutral pH the same protein could apparently be isolated (Fig. 1A).

To exclude the possibility that the binding of the 42-kDa protein to insoluble β-1,3-glucan or LPS resulted from aspecific interactions, the coelomic fluid was preincubated with soluble laminarin or soluble S-LPS from *S. typhimurium* or *E. coli* O55:B5, before incubation with curdlan or insoluble LPS. This treatment abolishes completely the binding of the 42-kDa protein to the glucan or LPS particles (Fig. 1, B and C). In contrast, neither cellobiose (Glcβ-1,4Glc) nor LPS from *S. minnesota* (Re) inhibits the binding of the 42-kDa protein to curdlan or LPS particles (Fig. 1, B and C). Thus, the binding of the 42-kDa protein from the coelomic fluid of *E. foetida* to β-1,3-glucan or S-LPS seems to be specific.

Cross-reactivity of Glucan- and LPS-binding Protein with Anti-CCF-1 mAb—Since the previously described cytolytic, hemolytic, and opsonizing protein CCF-1 from the coelomic fluid of *E. foetida* (19) had the same molecular weight as glucan- and LPS-bound material, this material was probed with the anti-CCF-1 mAb 12C9 (19). As shown on Fig. 1D, the 12C9 mAb recognizes the 42-kDa band corresponding to the glucan- or LPS-binding protein. Moreover, biotinylated laminarin binds to CCF-1 purified on anti-CCF-1 immunoaffinity column (not shown). These data suggest that the glucan- and LPS-binding protein and CCF-1 are identical proteins.

**FIG. 4.** Binding of anti-CCF-1 mAb or laminarin to rCCF-1. Blot showing rCCF-1 revealed with anti-CCF-1 mAb (A) or biotinylated laminarin (B). C, rCCF-1 stained with silver.
contrast glucose and saccharides bearing β-1,4- or β-1,6-glucosidic links are ineffective to block the interaction of laminarin or S-LPS with rCCF-1. Rough LPS mutants, particularly Re-, Rd-, and Re-LPS, do not inhibit the binding of laminarin or S-LPS to rCCF-1. Moreover, lipid A moiety of LPS from E. coli F583 Rd does not influence the binding of β-1,3-glucan or S-LPS to rCCF-1. Collectively, these results indicate that rCCF-1 binds both Glcβ-1,3Glc link and the O-antigen of LPS.

**Involvement of CCF-1 in Prophenoloxidase Activation in Earthworm Coelomic Fluid**—The activation of pro-PO system is based on the recognition of microbial glucans or LPS. It was thus interesting to test whether the glucan- and LPS-binding protein from the coelomic fluid of *E. foetida* trigger this defense mechanism. Since the activation of pro-PO is dependent on proteolytic enzymes (28, 29), the level of the activation was estimated as the difference of i-DOPA oxidation in the absence and presence of serine protease inhibitor. Coelomic fluid of *E. foetida* by itself causes only low level of oxidation of the substrate. However, in the presence of a triggering stimulus such as laminarin or LPS, the oxidation of i-DOPA by coelomic fluid proteins increases reaching a maximum between 6 and 8 h of incubation (not shown). As shown in Fig. 6, the yeast *S. cerevisiae* and the Gram-negative bacteria *S. typhimurium* LT2 (S) induce a dose-dependent activation of the pro-PO cascade. *E. coli* 055:B5 (S) and *E. coli* 0111:B4 (S) also trigger pro-PO activation in the coelomic fluid (not shown). In contrast, the induction of pro-PO activation by *S. minnesota* (Re) or the Gram-positive *B. firmus* is close to background. As few as 100 pg/ml laminarin or S-LPS from *S. typhimurium* are sufficient to trigger i-DOPA oxidation in the coelomic fluid. Other glucans bearing β-1,3-glucosidic link (curdlan, zymosan) could also trigger pro-PO activation, whereas glucose or saccharides with β-1,4-(cellobiose, lichenan) or β-1,6-glucosidic link (gentiobiose) are inactive (not shown). Finally, we observed that LPS fails to trigger i-DOPA oxidation in *E. foetida* coelomic fluid following removal of the O-antigen (Fig. 7). Lipid A is also unable to activate the pro-PO cascade.

To confirm that glucan- and LPS-binding protein CCF-1 is involved in the activation of pro-PO cascade, CCF-1 was removed from the entire coelomic fluid by preincubation with anti-CCF-1 mAb coupled to Affi-Gel (anti-CCF-1 mAb does not induce PO activity, not shown). CCF-1 removal results in a significant decrease of the oxidative activity of the coelomic fluid even in presence of yeast or Gram-negative bacteria (S) cell wall compounds (Fig. 8). The PO activity of the CCF-1-depleted coelomic fluid can be recovered by addition of rCCF-1. However rCCF-1 by itself does not trigger the oxidation of i-DOPA. Altogether, these results indicate that, upon interaction with yeast β-1,3-glucans or with the bacterial external

**TABLE I**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Laminarin binding</th>
<th>S-LPS binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-LPS (E. coli 055:B5)</td>
<td>30</td>
<td>6.5</td>
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<tr>
<td>Boiled S-LPS (E. coli 055:B5)</td>
<td>35</td>
<td>7.8</td>
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<tr>
<td>S-LPS (S. typhimurium)</td>
<td>42</td>
<td>9.8</td>
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<tr>
<td>Boiled S-LPS (S. typhimurium)</td>
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<td>10.8</td>
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<tr>
<td>Delipidized LPS</td>
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<td>1.0</td>
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<tr>
<td>Ra-LPS</td>
<td>NI</td>
<td>&gt;80</td>
</tr>
<tr>
<td>Rc-LPS</td>
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<td>NI</td>
</tr>
<tr>
<td>Rd-LPS</td>
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</tr>
<tr>
<td>Re-LPS</td>
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<tr>
<td>Lipid A</td>
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<tr>
<td>Laminarin (β-1,3-Glc link)</td>
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<tr>
<td>Curdlan (β-1,3-Glc link)</td>
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<td>7.2</td>
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<td>Cellobiose (β-1,4-Glc link)</td>
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<td>NI</td>
</tr>
<tr>
<td>Lichenan (β-1,4-Glc link)</td>
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<td>NI</td>
</tr>
<tr>
<td>Gentiobiote (β-1,6-Glc link)</td>
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<tr>
<td>Glc</td>
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</tr>
<tr>
<td>Mannitol (linear Glc)</td>
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<td>NI</td>
</tr>
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</table>

a Dose of inhibitor required to inhibit by 50% (ED_{50} in μg/ml) the binding of laminarin (50 μg/ml) to immobilized rCCF-1.

b Dose of inhibitor required to inhibit by 50% (ED_{50} in μg/ml) the binding of S-LPS from E. coli 055:B5 (10 μg/ml) to immobilized rCCF-1.

c Inhibitors were boiled 1 h to denature most protein and nucleic acid contaminants.

d NI, not inhibiting at 1 mg/ml.

e Contains α-mannan.

f Possible contaminants of laminarin.

**Fig. 6.** Activity of pro-PO cascade in the coelomic fluid by microorganisms, laminarin, and LPS. Coelomic fluid levels of l-DOPA oxidation are expressed as the mean of Δ_{max} values difference of the sample without and with protease inhibitor. Different doses of yeast *S. cerevisiae*, Gram-negative bacteria *S. typhimurium* (S), and *S. minnesota* (Re), Gram-positive *B. firmus*, laminarin, and S-LPS from *S. typhimurium* were used for activation of pro-PO. l-DOPA oxidation in presence of nonactivated coelomic fluid was considered as background level. S.D. did not exceed 15%.
Glucan-/LPS-binding Protein in Earthworm Coelomic Fluid

O-specific chain of LPS, CCF-1 may play a role in the pro-PO-activating system of *E. foetida* earthworms.

**DISCUSSION**

Extensive studies were carried out over the past 3 decades on the biochemical aspects of the defense system in invertebrates. It is now clear that invertebrates lack specific immunoglobulins, lymphocytes, or other features of the vertebrate adaptive immune system but possess innate defense components (30). One candidate for such immediate recognition and defense mechanisms is the prophenoloxidase (pro-PO) system that is often put in analogy with the alternate pathway of complement activation (1, 15, 31–34). The pro-PO-activating system was described in a large variety of invertebrates, but the activity level differs (1, 15). In particular, papers documenting the pro-PO system in annelids are more scarce (13, 16, 17), although a protein responsible for PO activity was described recently in oligochaetes (18).

In the present study we found that the coelomic fluid of *E. foetida* contains a 42-kDa β-1,3-glucan-binding protein which also clearly recognizes S-LPS. This protein is identical with previously described cytolytic protein CCF-1 (19, 22). Since recognition of microbial saccharides is an initial step of pro-PO activation, we focused on the possible involvement of CCF-1 in the initiation of pro-PO cascade. The pro-PO of *E. foetida* coelomic fluid is efficiently activated by yeast and Gram-negative bacteria (S) but not by Gram-negative (R) or Gram-positive bacteria. Moreover, cell wall constituents of yeast (β-1,3-glucans) or Gram-negative bacteria (S-LPS) also induce the oxidation of L-DOPA. When CCF-1 is removed from the coelomic fluid, the activation cascade is blocked. However, rCCF-1 possessing the same properties as the native one, i.e. reacting with anti-CCF-1 mAb, binding to β-1,3-glucans, and binding to S-LPS, restores the pro-PO activation of CCF-1-depleted coelomic fluid. These results suggest that the biological function of CCF-1 as polysaccharide-binding protein might be to trigger PO defense mechanism of earthworms confronted to yeast or Gram-negative bacteria.

In our experimental conditions, the activation pro-PO cascade could not be achieved in *E. foetida* cell-free coelomic fluid in the presence of Gram-positive microorganisms. However, the possibility that Gram-positive bacteria cell wall components such as peptidoglycan could trigger pro-PO activation in *E. foetida* is not excluded. Such activation may require particular physiological conditions that were not investigated in this study, for instance the digestion of peptidoglycan by lysozyme-like substance known to participate in earthworm defense mechanisms (35) or the presence of coelomocytes (36).

Lectins take part in invertebrate defense reactions by aggregating and opsonizing microorganisms (for review see Refs. 37 and 38). In this regard, we observed that CCF-1 agglutinates S Gram-negative bacteria, but not R Gram-negative bacteria or Gram-positive bacteria (not shown). Moreover, CCF-1 was reported to be involved in the opsonizing properties of the coelomic fluid (19). Hence besides its possible involvement in pro-PO activation, as a lectin, CCF-1 may serve as opsonin providing...
an efficient mechanism for phagocytosis during earthworm defense reaction.

CCF-1 seems to interact with the strain to strain variable O-antigen of LPS but not with the more conserved lipid A or R core. The biological significance of a lectin binding only a subset of Gram-negative bacteria is not known. Invertebrate defense molecules recognizing the lipid A or R core of LPS have been amply documented (37), but the occurrence of lectin binding the O-antigen of LPS is not unusual (39). Therefore, molecules other than CCF-1 may participate in the recognition of the lipid A or the R core of LPS and may cooperate with CCF-1 to a more efficient discrimination of pathogenic and commensal Gram-negative microbes in *E. foetida* coelomic fluid.

CCF-1 is a new protein as judged from data bank comparisons. However, a sequence motif exhibits homology with the bacterial β-1,3- and β-1,3,1,4-glucanases (24) and with the sea urchin β-1,3-glucanase (25). This motif is also present in two invertebrate proteins involved in innate defense mechanisms, namely the β-1,3-glucan-sensitive subunit α of coagulation factor G from horseshoe crab *L. polyphemus* (26) and the Gram-negative bacteria-binding protein from the silkworm *B. mori* (27). Although those two latter proteins display the highest homology in or near the putative catalytic site of the β-1,3- and β-1,3,1,4-glucanases, neither coagulation factor G nor Gram-negative bacteria-binding protein were shown to exert glucanase activity. Similarly we could not provide evidence of glucanase activity (40, 41) for native or rCCF-1 (not shown). One could, however, speculate that some amino acid residues of the conserved motif might be pertinent for saccharide recognition (42).

Despite their homology, CCF-1 and *Limulus* factor G present some important differences as follows. (i) Coagulation factor G binds β-1,3-glucans but not LPS (43). In contrast earthworm CCF-1 recognizes both. (ii) Factor G subunit β exerts proteolytic activity (26). However, although the coelomic fluid of *E. foetida* possesses high proteolytic activity and some proteases with a molecular mass of about 42 kDa were described (44–47), we failed to detect proteolytic activity for native and recombinant CCF-1 (not shown).

The glucan- and LPS-binding potential of CCF-1 and its possible participation in pro-PO activation, together with its cytolytic activity (19), suggest an involvement in worm defense mechanisms. The pleiotropic activities of CCF-1 is not surprising since molecules implicated in innate defense mechanisms of invertebrates exert various functions. For example, the antibacterial and hemolytic systems seem tightly connected since only the growth of bacteria bearing common epitopes with vertebrate erythrocytes is inhibited (30, 48–50). Moreover, it was described that at least one of the osonopizing proteins adsorbing on the surface of synthetic particles participates in hemolytic events (51). Finally components of the pro-PO cascade were described to have cytolytic, antibacterial, and opsonizing properties (2, 5, 7–9). All these studies illustrate the complexity of the invertebrate innate defense mechanisms.

Future investigations are in progress to elucidate the interaction between β-1,3-glucans or LPS and CCF-1. These studies may provide important insight into the mechanisms by which the toxic products of microbial infection may be controlled by *E. foetida* earthworms.

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