Studies on the Function of Peroxidasins in Innate Immune System in *C. elegans*

Jeong Hoon Cho†

Abstract

Peroxidasin is a unique member of peroxidase family in that it has extracellular matrix (ECM) motif as well as peroxidase activity. Peroxidasins are involved in consolidation the extracellular matrix during development and in innate immune defense. *C. elegans* has two functional peroxidasins, PXN-1 and PXN-2, and PXN-2 is known to contribute to innate immune system. However, it is not clear of PXN-1 function in innate immune system. Therefore, this study is focused on the function of PXN-1 and the relationship between PXN-1 and PXN-2 in innate defense system in *C. elegans*. When *pxn-1* was knocked down by RNAi, the worm turned to be more resistant to pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the enhanced resistance was abolished in *pxn-1pxn-2* double knock down. By contrast, *pxn-2* knock down showed strong er susceptibility to the pathogens. These results suggest that *pxn-2* can contribute the pathogen resistance and *pxn-1* can suppress the pathogen resistance. To confirm the idea, overexpression experiments were performed. Overexpression of *pxn-1* showed more susceptible to pathogens compared to the control and double overexpression of *pxn-1pxn-2* overcame the susceptibility of *pxn-1* overexpression to the pathogens. On the other hand, *pxn-2* overexpression made the worm more resistant to the pathogens and the resistance was maintained in *pxn-1pxn-2* double overexpression. The comparison of the susceptibilities to the bacterial pathogens in above mentioned constructs suggests that PXN-1 suppress the function of PXN-2 in defense against bacterial pathogens in *Caenorhabditis elegans*.

Keywords: Peroxidasin, Innate Immune System, Pathogen, *C. elegans*

1. Introduction

Peroxidasin (PXN) is a member of peroxidase family and consists of two distinctive regions, secretory N-terminal and peroxidase C-terminal region\(^{[1,2]}\). It was first discovered in *Drosophila* in 1994 and the unique structure suggested functions of extracellular matrix (ECM) consolidation, phagocytosis, and pathogen defense\(^{[1]}\). Later, in *Caenorhabditis elegans* (*C. elegans*) in vivo study, peroxidasin-2 is involved in ECM consolidation to reinforce collagen network\(^{[2,3]}\).

Peroxidasins are animal heme peroxidases some of which are involved in immune defense. The best-studied one is myeloperoxidases (MPO), generating hypohalous acid (HOCl) from H\(_2\)O\(_2\) and chloride to kill pathogens in mammalian system\(^{[4]}\). Moreover, SKPO-1 (ShKT-containing peroxidase) peroxidase has a critical role in innate immunity against *Enterococcus faecalis* in *C. elegans*\(^{[5]}\). In addition, according to Shi et al., peroxidasin is involved in murine lung host defense by recognizing gram-negative bacteria and by killing the bacteria\(^{[6]}\). The leucine repeats and immunoglobulin domains in N-terminal contribute the host-pathogen recognition and the peroxidase activity in C-terminal generates hypohalous acids to kill bacterial\(^{[6]}\).

There are two peroxidasin homologues, PXN-1 and PXN-2, in *Caenorhabditis elegans* (*C. elegans*). It is thought that *pxn-1* and *pxn-2* may have an antagonistic functional relationship in mechanosensory neurons in *C. elegans*\(^{[2]}\). A recent study suggests that PXN-1 regulate PXN-2 negatively in *C. elegans* neurodegeneration during aging\(^{[7]}\). However, it is yet to be studied how *pxn1* and *pxn2* interact each other in innate immunity. Therefore, this study focuses on how to affect *pxn-1* deficiency or overexpression on *pxn-2* function to pathogen resistance in *C. elegans*. 
2. Materials and Methods

2.1. Strains and Culture Conditions

Wild-type Bristol N2 strain and \( pxn-1 \) (\( ok785 \)) deletion mutant were obtained from the Caenorhabditis elegans Genetics Center (CGC) at the University of Minnesota. Breeding of \( C. elegans \) was carried out according to published protocols[8].

\( Bacillus subtilis \) (KACC10854), \( Staphylococcus aureus \) (KACC10196) and \( Pseudomonas aeruginosa \) (KACC10259) were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Korea.

2.2. Constructs and Transformation

Fire feeding vector (L4440) was ligated with the green fluorescent protein gene (\( gfp \)), \( pxn-1 \) genomic DNA (1.7kb, cut by BglII and SpeI) and \( pxn-2 \) genomic DNA (1.4kb, cut by BglII and KpnI) to obtain \( gfp \) RNAi, \( pxn-1 \) RNAi and \( pxn-2 \) RNAi bacterial feeding constructs. The RNAi constructs were introduced to HT115 (DE3) bacteria. To knock down \( pxn-1 \) and \( pxn-2 \) gene expression, the experimental worms were fed on RNAi transformed HT115 at 48 hours and their eggs were used for survival assay. RNAi had been fed continuously until the hatched eggs were grown to young adults (56 hours post hatching) as described in Kamath et al.[9].

To prepare the translational fusion constructs, \( pxn-1 \) and \( pxn-2 \) full length cDNA were ligated to heat shock promoters and fused with \( gfp \) to yield \( Phs::pxn-1::gfp \) and \( Phs::pxn-2::gfp \). The 10 ng/\( \mu l \) construct was injected into wild type N2 or \( pxn-1 \) (\( ok785 \)) mutant. 80 ng/\( \mu l \) of plasmid pRF4 containing a dominant gene (rol-6) was co-injected for isolation by their rolling pattern. Microinjection was performed as previously described[10]. GFP expression was detected using a fluorescent microscope system (80i-DS-Fi1, Nikon). For ectopic expression, the transgenic worms were subjected to heat shock at 30\( ^\circ \)C for 1 hour every 12 hours and followed by pathogen survival assays.

2.3. Survival Assay

Gravid worms were treated with hypochlorite and then washed with M9 buffer several times to synchronize. The survived eggs were used for the experiments. To measure survival rates, approximately 1000 individual eggs of individual genotypes were grown to young adults (56 h after hatching) at 20\( ^\circ \)C and the worms were transferred to a bacteria lawn plate either non-pathogenic \( Bacillus subtilis \) or pathogenic \( Staphylococcus aureus \) and \( Pseudomonas aeruginosa \). Subsequently, live worms in a specific plate were counted at every 6 hours.

2.4. Statistical Analysis

All survival assays against pathogenic bacteria, \( S. aureus \) and \( P. aeruginosa \) including non-pathogenic \( B. subtilis \) were calculated and differentiated by using the log-rank test of OASIS 2[11].

3. Results & discussion

3.1. \( pxn-2 \) Gene Knock Down Caused Pathogen Susceptibility, but \( pxn-1 \) Knock Down Gained the Resistance

To investigate functions of peroxidasins in \( C. elegans \) innate immunity, we performed a worm survival assay against two pathogenic bacteria, \( Staphylococcus aureus \) (\( S. aureus \)), and \( Pseudomonas aeruginosa \) (\( P. aeruginosa \)). In addition, non-pathogenic bacteria, \( Bacillus subtilis \) (\( B. subtilis \)), were tested for pathogen sensitivity as a control. In \( B. subtilis \) treatments, knock-down of \( pxn-1 \) (\( pxn-1 \) KD) and \( pxn-2 \) (\( pxn-2 \) KD) by using RNAi showed similar survival rates and they were not significantly different from the \( gfp \) control rate (Fig. 1A). In contrast, pathogenic bacteria, \( S. aureus \) and \( P. aeruginosa \), caused different responses in \( pxn-1 \) KD and \( pxn-2 \) KD worms. \( pxn-2 \) KD was 28% more susceptible to \( S. aureus \) than the control in the mean survival time (Fig. 1B). In \( P. aeruginosa \), \( pxn-2 \) KD survival time was 10% less than the control in the mean survival time (Fig. 1C). On the other hands, \( pxn-1 \) KD showed 13% enhanced resistance to the both pathogens compared to the control (Fig. 1B and C). Therefore, these results suggest that \( pxn-2 \) KD causes susceptibility and \( pxn-1 \) KD causes resistance to both pathogens. All three strains were more resistance to \( P. aeruginosa \) than to \( S. aureus \). The different susceptibility can be the result of cell wall structure difference between \( S. aureus \), a gram-positive and \( P. aeruginosa \), a gram-negative. Recently, peroxidasin can kill gram-negative bacteria by binding between immunoglobulin domain and lipopolysaccharide of bacteria and by generating hypohalous acids in murine lung defense[6].
3.2. Overexpression of pxn-2 Enhanced Resistance to the Pathogens

For further understanding of peroxidase-2’s role in innate immune defense, we examined survival rates of pxn-1 and pxn-2 overexpressing worms fed on the pathogens. Heat shock promoter was used for ectopic expression during pathogen treatment. pxn-1 and pxn-2 overexpressing worms were slightly sensitive to B. subtilis compared to the gfp controls (Fig. 2). As expected by the RNAi knock down data (Fig. 1), pxn-1 overexpression resulted in pathogen sensitivity; worms of pxn-1 overexpression showed 9% decreased mean survival time compared to gfp control to S. aureus and 13% less to P. aeruginosa (Fig. 2B and C). The overexpression of pxn-2 gained resistance against both pathogens, 22% and 14% longer survival time than the gfp control (Fig. 2B and C). These results conclude that pxn-2 overexpression can strengthen the innate immune defense, but pxn-1 overexpression restrict the defense. Therefore, pxn-2 may function on pathogen resistance and pxn-1 may restrict the resistance via interacting with pxn-2.

3.3. Double Deficiency of pxn-1 and pxn-2 Compensate the Resistant Effect on pxn-1 Knock Down to Pathogens

In addition to the above gene knock down and overexpression results, previous study showed that PXN-1 interacted with PXN-2 reversely in neurodegeneration of aged C. elegans. Therefore, pxn-1 can function on pxn-2 negatively in innate immune system. To test the relationship between pxn-1 and pxn-2, pxn-1 and pxn-2 gene double knock down were constructed and the survival rate was examined. pxn-1 mutant instead of pxn-1 RNAi was used in this experiment due to low efficiency of two different RNAi feeding [9]. For the control, pxn-1 mutant fed on gfp RNAi was examined. There was no significant difference between the mean survival hours of control pxn-1 (ok785) and pxn-1 with pxn-2 RNAi. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.

Fig. 1. Knock down of pxn-1 gene in C. elegans resists to the pathogens and pxn-2 knock down is sensitive to the pathogens. (A) Survival of pxn-1 and pxn-2 knock down worms to B. subtilis. (B) Survival of pxn-1 and pxn-2 knock down worms to S. aureus. (C) Survival of pxn-1 and pxn-2 knock down worms to P. aeruginosa. gfp RNAi, N2 treated with gfp RNAi; pxn-1 RNAi, treated with pxn-1 RNAi; pxn-2 RNAi, treated with pxn-2 RNAi. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.

Fig. 2. Overexpression of pxn-1 and pxn-2 results in the opposite responses against the pathogens. (A) Survival of pxn-1 and pxn-2 overexpressing worms to B. subtilis. (B) Survival of pxn-1 and pxn-2 overexpressing worms to S. aureus. (C) Survival of pxn-1 and pxn-2 overexpressing worms to P. aeruginosa. Phs::GFP, heat shock promoter combined with gfp; Phs::pxn-1::GFP, heat shock promoter with pxn-1 and gfp; Phs::pxn-2::GFP, heat shock promoter with pxn-2 and gfp. N > 600 individuals per each treatment. The survival rate is generated and differentiated by using OASIS 2.
pxn-1 RNAi, 42.74 and 43.3 hours respectively to S. aureus treatment and 48.58 and 47.81 hours to P. aeruginosa (Fig. 3B and C). However, the pxn-1 mutant fed on pxn-2 RNAi became sensitive to both pathogens, 36.18 mean survival hours to S. aureus and 39.61 hours to P. aeruginosa (Fig. 3B and C). These results suggest that additional pxn-2 knock down in pxn-1 mutant background eliminate the enhanced effect of pxn-1 mutant survival in innate immune system (Fig. 1). In addition, the results suggest that pxn-2 can affect pxn-1 reversely in innate immune defense.

3.4. The Double Overexpression of pxn-1pxn-2 Overcame Pathogen Susceptibility of pxn-1 Overexpression

Double knock down experiment suggested a negative interrelationship between pxn-1 and pxn-2 function in innate immunity. Thereafter, we confirm the idea by using double overexpressing worms against pathogens. At first, the amount of overexpression should be compensated. The used worms in this experiment were following: twice amount of gfp injection to N2 for the control (2x Phs::gfp), pxn-1 and gfp for pxn-1 overexpression (Phs::pxn-1::gfp + Phs::gfp), pxn-2 and gfp for pxn-2 overexpression (Phs::pxn-2::gfp + Phs::gfp) and pxn-1 and pxn-2 for pxn-1pxn-2 overexpression (Phs::pxn-1::gfp + Phs::pxn-2::gfp).

Consistent with the previous result (Fig. 2), pxn-1 overexpressing worms survived less than the control, 13% decreased mean survival time to both pathogens (Fig. 4B and C). pxn-2 overexpression increase the mean survival hours, 24% and 19% increase to S. aureus.
aureus and to P. aeruginosa (Fig. 4B and C). pxn-1pxn-2 double overexpression made the mean survival time longer than the control and pxn-1 overexpression, but the survival time was no significantly different from pxn-2 overexpression to S. aureus (Fig. 4B). In P. aeruginosa, pxn-1pxn-2 double overexpressing worms survived longer than the control and the pxn-1 overexpressing ones. However, the mean survival time of double overexpression was not significantly different from pxn-2 overexpression (Fig. 4C). Double knock down and double overexpression results reinforce the idea of reverse interrelationship between pxn-1 and pxn-2; pxn-1 can suppress the resistant effect of pxn-2 in innate immune defense in C. elegans. Peroxidasin is considered as a peroxidase with a secretory motif, which previous studies focused on its antioxidant activity. Recently, the maintenance of extracellular matrix structure and their actions on innate immunity has focused to develop efficient bacteria killing agents[6,12].

4. Conclusion

In Caenorhabditis elegans innate immune system, peroxidasin 1 may suppress the resistant role of peroxidasin 2 against pathogenic Staphylococcus aureus and Pseudomonas aeruginosa. Further research of peroxidasin is necessary for developing a potential bactericidal agent and fast and massive screening can be done by using C. elegans.

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References