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AUTOMATED ASSAY OF *Caenorhabditis elegans* WILD-TYPE AND CYSTATIN MUTANTS THRASHING BEHAVIOUR IN THE PRESENCE OR ABSENCE OF PLANT DERIVED CYSTEINE PROTEINASES (CPs)

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AUTHOR'S CONTRIBUTION

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

The intensive use and over dependence on synthetic anthelmintics for the treatment of nematode infection on only a few drugs with similar mode of action has put pressure on such drug candidates with resultant loss of potency due to development of resistance by target nematodes. Plant materials with promising quality and efficacy to substitute for current anthelmintics include the plant derived cysteine proteinases (CPs). Motility is an important indication of the effectivenes of a drug and is a characteristic of phenotype useful for high thoroughput screening of chemical and theraputic agents. This study determined the effect of cysteine proteinases on motility of *C. elegans* strains (wild type and cystatin null mutants) using the worm watcher device. Results show that motility of *C. elegans* was affected differently in PLS or papain. The effect of CP on motility of *C. elegans* strains was dependent on CP type, time of incubation and concentration of CP. Generally there was no significant difference ($P>0.05$) between mean motility of WT, *cpi-1* and *cpi-2* null mutant *C. elegans* in PLS when compared with PLS+E64 (control). There was a statistically significant ($P<0.05$) effect of papain dose on all the strains. Enzyme specificity on cuticle structural proteins might be responsible for difference in pattern of attack observed between papain and PLS. CP has potency for use as effective anthelminthic.

Keywords: Cysteine proteinases; anthelmintics; papain; cystatin; potency.

1. INTRODUCTION

Plant materials with promising quality and efficacy to substitute for current anthelmintics include the plant derived cysteine proteinases (CPs) found in paw-paw (*C. papaya*), pineapple (*Ananas comosus*) and Fig (*Ficus spp*) [1,2]. The CPs attack nematodes by mechanism that differs from all modes of action of current synthetic anthelmintics, whose modes of action range from neuromuscular transmission inhibition to blockage of metabolic pathways [3]. The intensive use of the synthetic anthelmintics and the dependence of treatment of nematode infection on only a few drugs with similar mode of action has put pressure on the drug candidates with resultant loss of potency due to development of resistance by target nematodes [4-6].

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The activity of CPs against parasitic nematodes has been demonstrated *in vivo* for nematode parasites of mice, sheep and pig [7-9].

For a chemical or substance to be used as a drug its mode of action needs to be understood [10]. Phenotypic screening of molecules with mice and other similar models have been the trend. For these models, cost of maintenance, beauracracy of animal licencing and difficulty in genetic manipulation of the models are some of the disadvantages that have limited their use [11]. There is need to use alternative cheap and easily genetically manipulated models in order to test other potential sources of anthelmintic drug candidates. *Caenorhabditis elegans* is a good candidate and has been used extensively in *in vitro* assays to screen the effect of drugs, chemicals or mutations on motility [12-14]. *C. elegans* is good candidate for such assays because, it is easy to maintain in the lab and also can be manipulated easily [15]. It is one of the organisms with a full developmental programme and also a well-characterized genome including mutants. *C. elegans* has a simple anatomy, transparent body, short prolific life cycle and small body size. It has been demonstrated that *C. elegans* resists the attack of CPs by deploying cystatin gene products (Ce-CPI-1, and Ce-CPI-2). Loss of these genes increases the susceptibility of *C. elegans* to CP attack [1,16].

Motility is an important indication of the effectivenes of a drug and is a characteristic of the phenotype useful for high thoroughput screening of chemical and theraputic agents [17]. The current trend in motility assay is manual method. Manual methods for motility assays are dependent on the observer and have been used to screen drugs and chemicals employed as therapeutic agents. Manual methods have limitations; they are time consuming, cannot be deployed to screen large numbers of worms and suffer from error due to human manipulation and interpretation [13]. To overcome the above limitations a fast automated measurement of nematode thrashing has been developed which is capable of measuring and analysing a 30 seconds movie in less than 30 seconds. The computer application uses an aglorithm to measure the thrashing of *C. elegans* by statistical analysis of the Covariance Matrix between sets of worm frames to determine the period of thrashing [13,17,18].

In this study, interest is to assay the effect of CPs on thrashing of *C. elegans* strains (wild type and cystatin null mutants) using the method of Buckingham and Sattelle (2009). Our aims are to determine the activity of CPs on the *C. elegans* motility and to develop a fast throughput method that can be deployed in screening candidate anthelmintics.

2. MATERIALS AND METHODS

2.1 Source and Maintenance of Nematode

The wild type *C. elegans* Bristol strain N2 was kindly supplied by Andrew Phiri of The University of Nottingham, while the cystatin null mutants [cpi-$1^{-/-}$ (ok1213) and cpi-$2^{-/-}$(0k1256)] were kindly donated by Dr Ian Duce of School of Biology, The University of Nottingham. The worms were cultured and maintained on nematode growth medium (NGM) at 15^{o}C under standard laboratory conditions on agar plates seeded with a lawn of *E. coli* OP50 strain. Nematodes were synchronized and maintained in agar plate and washed by the method described by Phiri et al. [1].

2.2 Preparation of Cysteine Proteinases (CPs)

Two preparations of cysteine proteinases used in this study were (1) purified papain, (2x crystallised aqueous suspension) from papaya, purchased from Sigma-Aldrich UK (product No. P3125), and (2) papaya latex supernatant (PLS) prepared as described by Buttle et al. [8]. The molar concentration of active enzyme was determined and standardized as described by Buttle et al. [8] by active site titration of enzyme with the specific inhibitor of cysteine proteinases, L-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (E64), (Sigma-Aldrich Ltd Dorset UK). For PLS, assay doses at a final enzyme concentration of 24 µM and 120 µM were tested against each strain of C. elegans while for purified papain, assay doses at a final enzyme concentration of 24 µM and 50 µM were tested against each strain of C. elegans. The control was CPs+E64 (cysteine proteinases inhibited with E64).

2.3 Motility Assay

Early *C. elegans* adults (4^{th} moult) were used in this assay. Ten worms were pipetted into each well of a flat bottom 96 well plate (Costar®). Doses of CPs or CP+E64 were introduced into each well with the aid of a multi channel pipette. The experiment was performed as described by Buckingham and Sattelle, (2009) by placing the 96 well plate on the stage of worm watcher device. The thrashing movement of the *C. elegans* strains were assayed with or without papain or PLS over 1 h. This method computationally measured worm movement index in each dose of CP to determine the effects of the CP doses on the worms contrasted with the control. Each treatment was replicated six times for each *C. elegans* strain.

2.4 Statistical Analysis

Mann Whitney test was used to compare mean motility in treatments against the control (CP+E64). For the effect of CP on thrashing of worms, mean motility in each dose was compared with the mean motility in CP+E64. For effect of time of application, mean motility in periods of 1 and 2 h were compared to mean motility at time zero (t0). Where there was a significant difference between treatment and CP+E64 (control) for a particular CP type, we went on to compare the effects on the three *C. elegans* strains. In all the analyses the ascribed threshold significance level was set at P=0.05.

3. RESULTS

Table 1 shows the summary of statistical differences between mean motility of *C. elegans* strains in PLS or papain compared to CP+E64 (inhibited enzyme used as control). There was no significant difference between mean motility of worms at different concentrations/time of incubation in PLS when compared with the control (CP+E64) except for *cpi-2* strain after 2 hrs incubation in 120 µM PLS.

For purified papain, the study observed that motility of worms strains varied at different treatment levels and time of incubation when compared with mean motility in papain+E64, indicating a profound effect of purified papain against motility of all worm types. There were significant differences (P=0.05) between the mean motility of worm strains in papain compared with mean motility in the control (papain+E64) after incubation for either 1 h or 2 h respectively. The only exception was mean motility of *cpi-1* in 24µM papain, which was not statistically significant when compared to mean motility in papain+E64. At 24 µM papain, for WT verses *cpi-1,* analysis shows that *cpi-1* was less susceptible (P=0.04). Similarly at the same 24 µM concentration of papain, analysis of WT verses *cpi-2* did not show any statistically significant difference (P=0.18) between the motility of the two *C. elegans* strains, indicating that 24 µM papain affected WT and *cpi-2* in a similar pattern. Also at 24 µM, *cpi-1* was also less susceptible to papain attack when compared with *cpi-2* (P=0.01).

Fig. 1 shows the mean motility of WT, *cpi-1* and *cpi-2* at 24 and 120 µM concentrations of PLS. It was observed that the effect of PLS at a dose of 24 µM on motility of either WT, *cpi-1* or *cpi-2* did not show any significant difference when compared to mean motility of worms in PLS+E64 except the motility of *cpi-2* in 120 µM PLS.

Fig. 2 shows the mean motility of *C. elegans* strains in papain. There was dose effect when mean motility at 24 µM was compared with mean motility at 120 µM. Generally the motility of worms drastically declined with time in all treatment level when compared with the mean motility in papain+E64 at time 0. The rate of decline in motility was dependent on concentration of papain. Loss of motility increased from lower dose of 24 µM and increased more in high dose of 50 µM papain.

Table 1. Summary of the statistical differences between mean motility of *C. elegans* strains in either PLS or papain compared to mean motility in CP+E64 (inhibited enzyme used as control)

	PLS			Papain		
Worm type	**Conc. of PLS (µM)**	**Is treatment vs PLS+E64 Significant?**		**Conc. of papain (µM)**	**Is treatment vs Papain+E64 significant?**	
		60 min	**120 min**		**60 min**	**120 min**
WT	24	ns (P=0.3095)	ns (P=0.8182)	24	** (P=0.0087)	** (P=0.0049)
	120	ns (P=0.1320)	ns (P=0.0611)	50	** (P=0.0028)	** (P=0.0022)
CPI-1	24	ns (P=0.6991)	ns (P=0.3095)	24	ns (P=0.4848)	* (P=0.0411)
	120	ns (P=0.4003)	ns (P=0.0611)	50	** (P=0.0022)	** (P=0.0022)
CPI-2	24	ns (P=0.8182)	ns (P=0.5887)	24	* (P=0.0260)	** (P=0.0022)
	120	ns (P=0.1320)	** (P=0.0022)	50	** (P=0.0022)	** (P=0.0022)

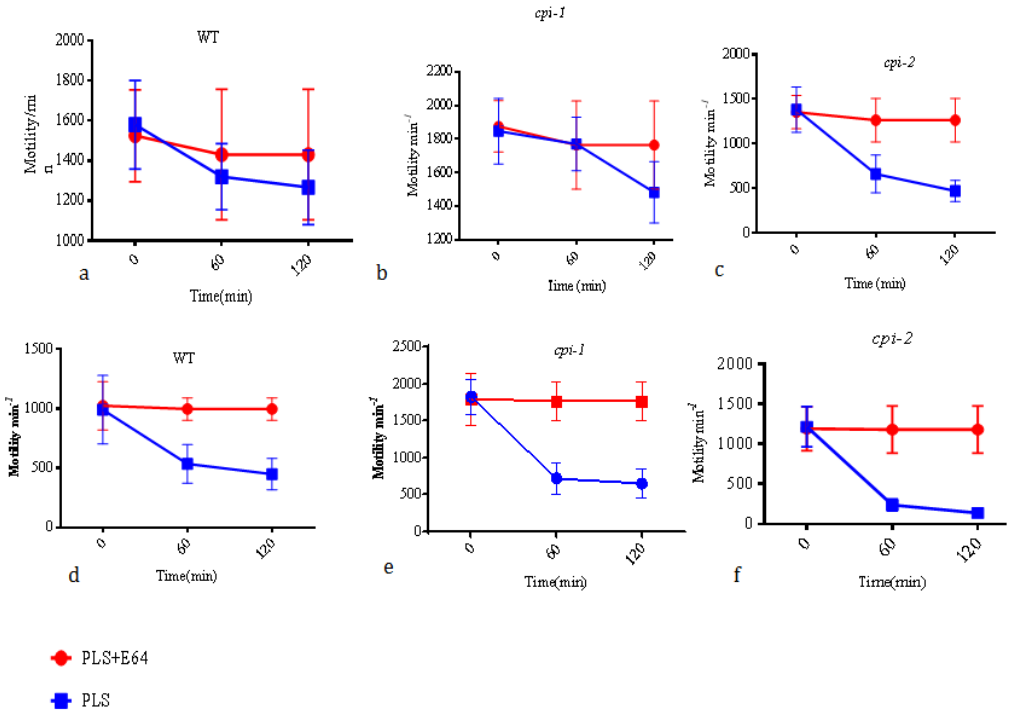

Fig. 1. Motility of WT, *cpi-1* and *cpi-2 C. elegans* strains in different concentrations of PLS

Fig. 1a-c show the motility of C. elegans strains in 24 µM PLS whereas d-f are showing motility in 120 µM. The motility of C. elegans was affected when incubated with either concentration of PLS. The effect was slight at 24 µM concentration of PLS especially for cpi-1 that seem to resist the PLS when incubated for 60 min. Error bar represent the SEM

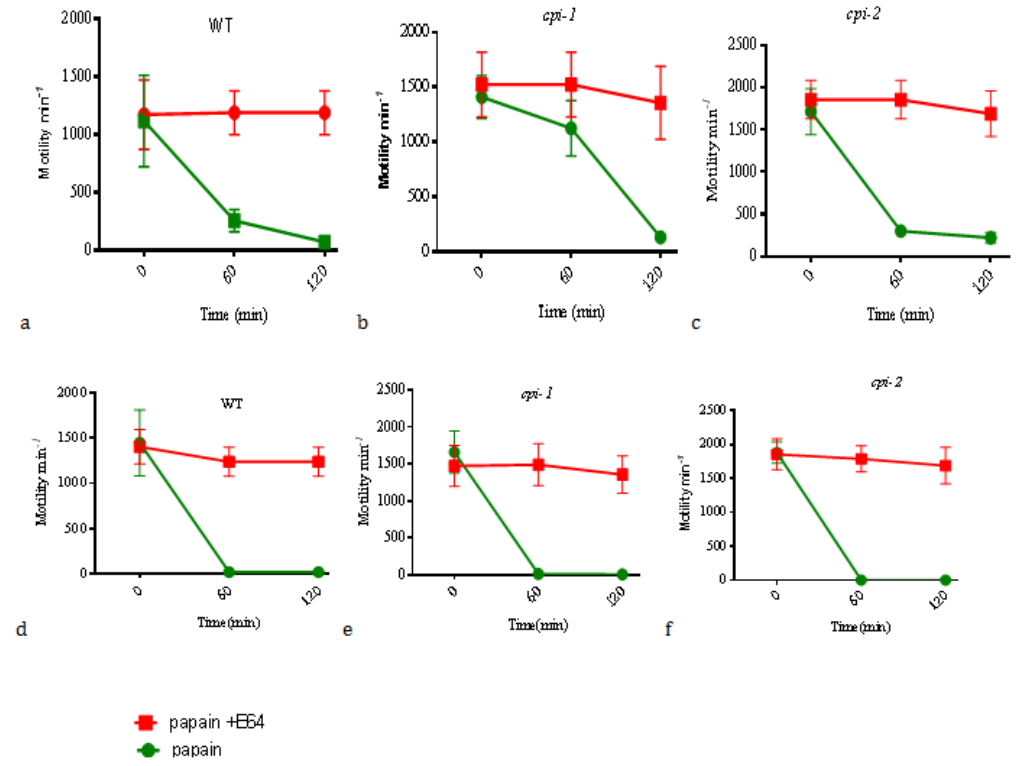

Fig. 2. Mean motility of *C. elegans* strains in concentrations of papain over time

Graph a-c showed the motility of WT, cpi-1 and cpi-2 in 24 µM papain whereas d-f is the motility of the worms in 50 µM papain. The error bar represents the ±SEM for each treatment level

4. DISCUSSION

This study found that the effect of CP on motility of *C. elegans* strains was dependent on CP type, time of incubation and concentration of CPs. Generally there was no significant difference (P>0.05) between mean motility of WT, *cpi-1* and *cpi-2* null mutant *C. elegans* in 24µM PLS when compared with PLS+E64. Also there was no statistical difference between the motility of WT and *cpi-1* at 120µM PLS (Table 1) however, *cpi-2* declined more in motility at this concentration of PLS when compared to PLS+E64 (Table 1).

Our study also found that purified papain affects *C. elegans* strains more than PLS. Worm motility was affected in all the concentrations of papain used when compared with papain+E64. Incubating worms in papain for 1 h or 2 h drastically affected the motility of the three strains of *C. elegans* except *cpi-1* that seems to be less susceptible at 24 µM papain after 1 h incubation.

CPs cause cuticular damage and mortality to many parasitic nematodes [19-23] and cestodes [24,25] and *C. elegans* [1]. The damage affects the motility of the worms when the integrity and function of their cuticles are lost. Vulnerability of parasitic worms to CP attack seems to be different from that of the free-living ones [1,26]. It was observed that the minimum concentration of CPs from plant source that can kill parasitic nematodes is 200 µM [20,27], but was found not to affect the motility of wild type (Bristol N2) *C. elegans* because WT *C. elegans* deployed cystatins to inhibit the activity of CPs [1,28]. Cystatin, a cysteine protease inhibitor, with an immune-regulatory role in parasitic nematodes [29,30] has been suggested to be deployed for protection by free living nematodes against exogenous CPs from bacteria, fungi and decaying plant material [1]. The presence of this array of protease inhibitors was thought to be a physio-immunological adaptive mechanism to withstand the changing chemical environment in which they dwell [31]. In *C. elegans*, two genes- *Ce-cpi-1* (K08B4.6) and *Ce-cpi-2a* (R01B10.1) encode for two cystatins (Ce-CPI-1 and Ce-CPI-2) known to function in moulting, ecdysis and oogenesis [30,31] and in wild type *C. elegans* the cystatins are deployed to inhibit the activity of CPs [1] and probably the inherent resistance allowed the worms some level of thrashing when incubated in CP medium.

Our data suggest that wild type *C. elegans* resistance to the concentrations of PLS used in this study was not very different from *cpi-1* and *cpi-2* mutants. The data from PLS is contrary to our hypothesis that *cpi-1* and *cpi-2* are more susceptible than WT. Our assay was unable to detect a difference in vulnerability between strains. Phiri et al (2014) were unable to observe any visible changes in the motility of WT after incubating in 120µM and up to 3000µM PLS for 3 h and concluded that WT was able to resist PLS because of secretion of cystatin. In the *cpi-1* and *cpi-2* mutant, active cystatin production has been eliminated which suggest that, the two cystatin mutants would be expected to quickly succumb to CP attack at high concentration (120µM) of PLS. we suspect that the pH status of the cuticle surface [1], and enzyme specificity, may be other factors affecting the PLS activity on nematode cuticle but this supposition needs to be further investigated. The worm epicuticle is covered by negatively charged glycoproteins surface coats [32] that might help to bind and aggregate the CPs, all of which are basic enzymes [33]. In our other study [26] SEM showed that cuticular damage was visible between 10 to 30 min in dead worms and there was no visible difference between the cuticular damage done by 1µM papain to either WT, *cpi-1* or *cpi-2* when incubated in same concentrations of PLS.

The other possible source of difference between our data and that reported elsewhere [1] could be in the choice of method of assessing worm thrashes. Manual methods are subjective, prone to error and do not adequately address subtle thrashing differences in healthy worms [1,13,34]. Unlike the manual method, an automated worm watcher used a computer vision that distinguishes the worm from its background, estimated the shape and determined the body angle of the worm from which the thrashes are calculated [13]. The subtle movement of worms that might not be noticed in manual method was captured by the 'worm watcher'. Therefore the sensitivity of the 'worm watcher' combined with the different densities (numbers) of worm in the replicates might be a source of variability.

Our findings also suggest that CP type affected the motility of *C. elegans* strains differently. Papain acutely affected the motility of all the strains when compared to PLS (Table 1). Papain is a purified CP and has less contamination than PLS. PLS is an unrefined mixtures of CPs, chymopapain, glycyl endopeptidase, caricain as well as papain (in order of decreasing abundance) [35]. The four enzymes constitute the CP activity in papaya latex [35,36]. The specificity of this CPs may be responsible for the difference in the degree of attack between PLS and papain. The enzymes in PLS will cleave different peptide bond targets on the cuticle structural proteins compared to papain alone. For instance papain prefers glutaminic acid, proline, leucine, arginine, glutamine, glutamine, arginine or aspartic acid at P1, P2, P3, P4,

P1' P2' P3' and P4' respectively whereas the most abundant CP in PLS, chymopapain [37] cleaves most efficiently at alanine, glycine, valine, arginine, and leucine at P1, P2, P3, P4, P1' and P4' respectively [38-40] (the cleavage 'hit' map for CP can be found here http://merops.sanger.ac.uk/cgi-bin/pepsum?id=C01.001).

The decline in motility of worm types incubated in papain as observed in this study was caused by papain. There was a significant difference between treatment and papain+E64 control (P=0.05). The loss of motility was also concentration and time dependent especially when the worms were incubated in papain. Drastic loss of motility in papain was recorded when worm types were incubated for 2 h as worm motility declined to zero in all the *C. elegans* strains. Healthy worms thrash happily in a non-toxic environment [41-43], such thrashing behaviour is impaired when worms are incubated in drugs or toxic medium [44, 45]. Immobilisation of *C. elegans* incubated in CPs is due to damage to the cuticles which function to protect the worms as well as aid to bring about motility of the animal [32]. Damage due to PLS or papain on nematodes and cestodes has been shown to be dependent on the activity of CPs [1,9,26,27,46, 47]. The mechanism of attack by CPs on nematode and cestode is by digestion and degrading the structural proteins, which confer integrity to the cuticle. Loss of the structural proteins leads to loss of integrity, motility and finally death of the nematode.

The data presented here also compared the automated method of assessing *C. elegans* motility in CPs with the manual method and has not totally agreed with findings elsewhere [1] which found that wild type *C. elegans* decline in motility in PLS was significantly slower from that of the cystatin null mutants. This study found generally that there was no statistically significant difference between the mean motility of worm types incubated in PLS (P=0.05). However, significant difference existed between WT verses *cpi-2* at the highest concentration of PLS (P=0.0115), indicating that *cpi-2* was more susceptible than WT.

5. CONCLUSION

This study has shown that motility of *C. elegans* is affected differently in PLS or papain. Motility of the three strains *C. elegans* was affected by exposure to papain, in a concentration, time- and CP type-dependent manner. Papain affected the motility of *C. elegans* and was more effective than PLS suggesting importantly that different CPs may have different potencies in different worms, so a good idea to have a mix such as PLS. PLS works well with parasitic worms. Thrashing of all the strains of *C. elegans* was reduced to zero at the highest concentration of 50 µM papain after 2 h incubation. Enzyme specificity on cuticle structural proteins might be responsible for difference in pattern of attack observed between papain and PLS.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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