Published in final edited form as:

Methods Enzymol. 2011 ; 499: 283–299. doi:10.1016/B978-0-12-386471-0.00014-6.

Using *C. elegans* to Identify the Protease Targets of Serpins *In Vivo*

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Abstract

Most serpins inhibit serine and/or cysteine proteases, and their inhibitory activities are usually defined *in vitro*. However, the physiological protease targets of most serpins are unknown despite many years of research. This may be due to the rapid degradation of the inactive serpin:protease complexes and/or the conditions under which the serpin inhibits the protease. The model organism *Caenorhabditis elegans* is an ideal system for identifying protease targets due to powerful forward and reverse genetics, as well as the ease of creating transgenic animals. Using combinatorial approaches of genetics and biochemistry in *C. elegans*, the true *in vivo* protease targets of the endogenous serpins can be elucidated.

1. Introduction

Apart from a few human extracellular serpins, the *in vivo* targets for most serpins have yet to be identified. This is particularly true for the intracellular serpins, whose natural *in vivo* serpin:protease complexes seem to be rapidly degraded, in part by the proteasome (Hirst *et al.*, 2003; Luke, C. J. and Silverman, G. A., unpublished data). Additionally, another hypothesis to explain the technical difficulties associated with serpin target identification is that the serpin and protease do not coexist within the same location within the cell until an external signal or stress is applied (e.g., Luke *et al.*, 2007). Thus, complexes can only be isolated under certain environmental and/or genetic conditions. Until recently, the genetic tools for phenotypic analysis of serpins in mammalian cell culture have been unavailable, and traditional pull-down techniques by coimmunoprecipitation have yielded little information. The use of model organisms to identify serpin function and protease targets has overcome some of the obstacles of human cell culture systems (Silverman *et al.*, 2010). One such model, *Caenorhabditis elegans*, was the first multicellular organism to have the entire genome sequenced (*C. elegans* Sequencing Consortium, 1998). From this data, the *C.*

C. elegans genome was found to contain no extracellular but nine intracellular serpin sequences of which six were functional protease inhibitors (Luke *et al.*, 2006; Pak *et al.*, 2004; Whisstock *et al.*, 1999). Although these serpins are more evolutionarily conserved at the amino acid level to each other than to mammalian intracellular serpins (Luke *et al.*, 2006; Silverman *et al.*, 2001), there is *in vitro* functional homology to many mammalian intracellular serpins (Luke *et al.*, 2007; Pak *et al.*, 2004, 2006). The genetic controllability of *C. elegans*, as well as its short life cycle, ease of cultivation and transparent body, makes this organism an ideal tool to identify the physiological protease targets of the intracellular serpin subfamily.

C. elegans is a transparent, free-living, androdioecious nematode consisting mostly of a nervous system, muscle, intestine, and reproductive system. It is the simplest multicellular model organism routinely used in the laboratory. The worm has a short life cycle going from egg to adult in 3.5 days at 20 °C and is relatively inexpensive to maintain and grow. Despite the extreme morphological differences, ~35% of the genes in *C. elegans* have homology with human genes and most of the fundamental cellular biological processes are conserved. The most beneficial tool available to the *C. elegans* researcher is the genetic tractability of this model organism. Both forward and reverse genetic, as well as transgenic approaches, can be used to understand protein function *in vivo*. Although less often utilized by *C. elegans* researchers, the ability to rapidly and easily generate transgenic animals (see Chapter 13) also makes this organism ideal for biochemical approaches aimed at identifying protein:protein interactions within a multicellular organism.

1.1. General reagents used for nematode growth and maintenance

Unless otherwise stated, we use the following reagents for the growth and maintenance of the *C. elegans* strains (Brenner, 1974).

1.1.1. Preparation of nematode growth medium (NGM)—Standard NGM is prepared in the following manner:

1. NGM base (3.0 g/l NaCl, 20.0 g/l Bacto-Agar, 2.5 g/l Bacto-peptone) is sterilized using an autoclave on a liquid cycle.
2. After sterilization, the media is then cooled to 55 °C in a water bath.
3. The following sterile solutions are added in the order shown below. Be sure to mix the flask thoroughly between each addition. These solutions must be added after autoclaving to prevent precipitation.
 - 1.0 ml/l cholesterol (5 mg/ml in 95% Ethanol; Sigma–Aldrich)
 - 1.0 ml/l 1 *M* MgSO₄
 - 1.0 ml/l 1 *M* CaCl₂
 - 25.0 ml/l 1 *M* KPO₄ buffer pH 6
4. Media is then dispensed into non-vented petri dishes (Tritech Research).

1.1.2. Preparation of OP50 for nematode food stocks—The laboratory food source of *C. elegans* is the *Escherichia coli* strain OP50. To grow OP50, we use the following protocol:

1. From a frozen glycerol stock, OP50 is streaked onto an LB agar plate and allowed to grow at 37 °C overnight.
2. A single colony from this plate is used to inoculate 10 ml of LB broth and grown overnight at 37 °C with shaking at 200 rpm.
3. 1 L of LB broth is inoculated with the 10 ml overnight culture and grown at 37 °C in an orbital shaker at 200 rpm until an $OD_{600} \approx 0.5$ is reached (this usually takes 5–6 h).
4. The OP50 is then centrifuged at $6000\times g$ for 5 min.
5. The supernatant is discarded and the resultant pellet is resuspended in 100 ml sterile M9 buffer (42.3 mM Na_2HPO_4 , 22.0 mM KH_2PO_4 , 85.6 mM NaCl, 1 mM $MgSO_4\cdot 7H_2O$).
6. The OP50 is then washed a further two times with 100 ml of sterile M9 buffer.
7. After the final wash, the pellet is resuspended in 100 ml of sterile M9 buffer plus 25% glycerol.
8. The OP50 is then dispensed into aliquots and frozen at $-80\text{ }^{\circ}\text{C}$ until needed.
9. When required, thaw the aliquot of frozen OP50 and pellet the bacteria by centrifugation at $6000\times g$ for 15 min.
10. The supernatant is discarded and the pellet is washed three times with 6 ml of sterile M9 Buffer. The OP50 is now ready to use.

2. Methods for Identifying the Targets of Intracellular Serpins in *C. Elegans*

Both biochemical and genetic approaches can be used to study the protein targets of serpins. Figure 14.1 depicts a flow chart of the scheme to identify protein targets. Both methodologies start with analyzing the genome. Using basic local alignment search tool (BLAST) analysis, *C. elegans* has nine intracellular serpin genes at the nucleotide level (Pak *et al.*, 2004; Whisstock *et al.*, 1999). Further analyses of cDNAs amplified from total RNA and phylogenetic analyses show that of these nine, only five probably serve to function as *bona fide* protease inhibitors; *srps-1*, -2, -3, -6, and -7 (Luke *et al.*, 2006; Pak *et al.*, 2004; Whisstock *et al.*, 1999). Although these serpins are more closely related to each other than to any of the mammalian serpins and, using phylogenetic analysis, form their own clade (clade L; Silverman *et al.*, 2001), BLAST searches directed exclusively against the human genome reveal that they have primary homology to different human serpins (Table 14.1). However, based on their *in vitro* inhibitory profiles, their functional homologues may be very different. For example, SRP-2 most closely resembles human SERPINI1 (Neuroserpin) at the primary amino acid level. SERPINI1 is an extracellular serpin and inhibits tPA, uPA, and plasmin (Osterwalder *et al.*, 1998), whereas in *C. elegans*, SRP-2 is an intracellular serpin that inhibits granzyme B and lysosomal cysteine peptidases (Pak *et al.*, 2004).

Therefore, SRP-2 is unlikely to be a functional homologue of SERPINI1, and only genetic and biochemical analyses will reveal if there is a human serpin with similar function to SRP-2.

2.1. Identifying serpin targets using genetics

The powerful genetic toolbox of *C. elegans* is one of the main attractions to using it as a model system. Even before a worm is handled, the powerful online database, Wormbase (www.wormbase.org), can provide a wealth of information for each specific serpin gene, such as genomic position, expression profiles, nucleotide and protein sequences, phenotypic information by RNAi and reagents available. Some of the reagents that can prove to be most useful for studying protein targets of serpins are knockout alleles. There are two centers which are dedicated to deleting all 19,735 protein coding genes: the *C. elegans* Gene Knockout Consortium (<http://celeganskoconsortium.omrf.org/>) and the National Bioresource Project for the Experimental Animal Nematode *C. elegans* (<http://www.shigen.nig.ac.jp/c.elegans/index.jsp>). For the five serpin genes, there is at least one knockout allele (Table 14.1). These knockout alleles have been deposited into a central resource: the Caenorhabditis Genetics Center (CGC; <http://www.cbs.umn.edu/CGC/>). The *C. elegans* strain containing the allele can be obtained through this central resource. However, most alleles are generated through random mutagenesis techniques, such as EMS (Brenner, 1974). These techniques will often cause mutation in other genes within the same genome and thus should be eliminated through backcrossing the mutant allele against the wild-type background and selecting for the serpin knockout allele by single worm PCR. These methodologies have been published previously by *C. elegans* genetic experts in Hess *et al.* (2006).

2.1.1. Phenotypic analysis—Identification of phenotypes associated with serpin knockouts in *C. elegans* can lead to insights into the targets of these genes. One approach to study protein function is to investigate the behavior of mutant animals in which one or more proteins are mutated to cause a visible macroscopic or microscopic phenotype. In such studies, it is necessary to characterize the general health of mutant animals relative to wild-type (N2) animals by carrying out longevity, brood size, and postembryonic development assays. These assays are carried out under standard conditions (Brenner, 1974), and the proper positive control is a population of the N2 Bristol strain that is tested under identical conditions. Each assay is repeated $n = 3$ times.

2.1.1.1. Longevity assay: Under standard laboratory conditions and at 20 °C, N2 *C. elegans* survive an average of 2–3 weeks (Brenner, 1974). In many cases, protein mutations may not have adverse effects on longevity under standard laboratory conditions, but longevity may be affected by subjecting mutants to environmental stress factors. For example, in the case of *srp-6* null animals, the median survival is ~1 min after immersion in water, whereas N2 animals survive for greater than 30 min under the same conditions (Luke *et al.*, 2007). Longevity has been described previously (Klass, 1977).

1. A population of 25 late L4 worms of a particular worm strain is placed on an NGM plate seeded with OP50.

2. The worms are transferred on a day-to-day basis to seeded NGM plates. Each day the percentage survival is recorded until the population is depleted. The data can be represented on a Kaplan–Meier survival curve, and longevities of various worm strains can be analyzed using a log rank test.

2.1.1.2. Development assays: *C. elegans* adjusts its metabolism to survive a range of temperatures from 16 °C to 25 °C under standard laboratory conditions. At 20 °C, the embryo develops through its four larval stages, L1 through L4, to become an adult after 3.5 days. At 15 °C, the life cycle is increased to 5.5 days, and at 25 °C, it is decreased to 2.5 days. At 25 °C, *srp-2* null mutants develop at a comparable rate to N2 worms, while *srp-2*-overexpressing worms are developmentally delayed due to the subjects undergoing larval arrest and slow development (Pak *et al.*, 2004).

Postembryonic development assays have been described previously by Larsen *et al.* (1995).

1. A synchronized population of 25 young adult worms are placed onto a seeded NGM plate and allowed to lay eggs for 2 h at the required temperature, after which the adults are removed.
2. The developmental stage of the progeny is scored after 72 h at 20 °C or after 48 h at 25 °C. N2 worms develop to the adult stage and/or L4 larvae after this time period. Slowly developing worms will remain in the L1–L3 stages and will eventually develop to the L4 or adult stage after this time period, while larvally arrested worms will not develop into adults.

2.1.1.3. Brood size: *C. elegans* are classified as androdioecious nematodes, where males occur naturally one in 1000. Each N2 hermaphrodite lays approximately 300 eggs by self-fertilization and this facilitates mass culture of the organism. The brood size of a worm strain is determined by summing the total number of progeny produced by a worm during its fertility period (Singson *et al.*, 1998).

1. Ten late L4 worms are individually placed onto seeded NGM plates on sequential days until the fertility period comes to an end. At 20 °C, the fertility period is 5 days, and at 25 °C, it is 4 days.
2. The number of eggs laid by a single subject is determined on each day or over the entire fertility period.

2.1.1.4. Tissue-specific phenotypes: The physiological processes of *C. elegans* can be macroscopically divided into seven major systems: epithelial, nervous, muscular, excretory, coelomocyte, alimentary, and reproductive. To study the functions of a protein in *C. elegans*, it is necessary to know the expression profile of the protein of interest. One method to do this is to tag the protein with a fluorescent marker protein and to create transgenic worm strains where the protein is expressed under its natural promoter. Often times, the system can be further reduced to study the function of the protein in a specific organ system. *C. elegans* offers the advantage of driving expression of genes under tissue-specific promoters. For example, SRP-6 has been shown to be expressed in intestinal cells, socket cells, vulva cells, and the pharyngeal/intestinal valve, but its function in intestinal cells can

be studied by driving its expression under an NHX-2 promoter as described previously (Nehrke and Melvin, 2002).

2.1.2. The use of RNAi by feeding to study protease targets—Once a phenotype has been found, the protease targets of the serpin can be inferred by the suppressor of the phenotype using RNA-mediated interference (RNAi) of specific protease genes. RNAi was first described in *C. elegans* (Fire *et al.*, 1998; Guo and Kemphues, 1995), a process in which introduction of double stranded RNA (dsRNA) results in the targeted silencing of a specific cognate gene. Advances in the underlying mechanism of RNAi (Grishok, 2005; Wang and Barr, 2005), and its experimental uses as well the availability of the complete genomic sequence of *C. elegans* (Consortium, 1998), have facilitated the functional analysis of both limited and large-scale gene sets involved in numerous biological pathways (Boutros and Ahringer, 2008; Kamath and Ahringer, 2003; Maine, 2008; Sugimoto, 2004). It is now common for groups to employ large-scale RNAi studies to systematically examine large sets of genes for specific knockdown phenotypes. Thus, RNAi is a reverse genetic approach where the examination of a particular gene is carried out by the knowledge of a specific sequence rather than by the presence of a mutant phenotype. In *C. elegans*, dsRNA can be introduced in four ways: injection of dsRNA into worms (Fire *et al.*, 1998), feeding animals with bacteria that produce a specific dsRNA (Kamath *et al.*, 2001), direct soaking of worms in solution containing the dsRNA (Tabara *et al.*, 1998), and the induced *in vivo* production of a specific dsRNA via an exogenous promoter (Tavernarakis *et al.*, 2000).

When using an RNAi-based approach, a key aspect of the study design is to consider the method of dsRNA delivery to the animals. Among dsRNA delivery methods, the feeding method is well suited to examine a potential large number of RNAis applicable to an entire library. The feasibility of inducing RNAi by feeding bacteria-expressing dsRNA specific for a gene of interest has been well characterized in *C. elegans* (Kamath *et al.*, 2001; Timmons and Fire, 1998). Once ingested by the worm, the dsRNA is processed and systemically spread, resulting in an RNAi-specific phenotype in both the original dsRNA recipients as well as subsequent progeny (Wang and Barr, 2005). Thus, advantages of RNAi by feeding include the ability to introduce dsRNA to a large number of animals at the same time, the ability to introduce dsRNA in a developmental stage-selective manner, and the ability to examine RNAi effects on successive generations.

The techniques used for RNAi in *C. elegans* have already been described in great detail (Kamath and Ahringer, 2003; Wang and Barr, 2005). To perform a large, systematic RNAi screen, a gene library is necessary to provide the template for the synthesis of a specific dsRNA. Gene libraries can be constructed based upon the use of genomic sequence or cDNA libraries. Specifically for the feeding method, the Ahringer RNAi library contains ~17,000 bacterial clones, covering ~87% of the *C. elegans* genome and has been employed in many large-scale and genome-wide RNAi screens (Fraser *et al.*, 2000; Kamath *et al.*, 2003; Timmons and Fire, 1998). The bacterial vector, L440, contains a genomic PCR fragment corresponding to the gene of interest flanked by T7 promoters in opposite orientations. The PCR fragment-containing plasmid is transformed into the *E. coli* host HT115(DE3) which is both RNase III deficient and contains an IPTG-inducible T7 polymerase.

The major application of using this methodology is to screen for and identify genes involved in a specific biological process or pathway. Compared to forward genetic screens, the major advantages of using an RNAi-based approach are the systematic nature by which genes in a particular pathway can be analyzed, and the speed by which the connection between gene identity and phenotype can be made. In addition to large-scale studies in which RNAi knockdown is performed throughout the entire genome, it is also possible to examine specific subsets of genes in a more targeted fashion.

Using this RNAi screening approach, we have begun to try to isolate specific targets of intracellular serpin activity. Specifically, we have employed the MEROPS (<http://merops.sanger.ac.uk>) peptidase database to identify ~400 peptidases found within the *C. elegans* genome to screen as potential serpin regulatory targets. In particular, we have begun to identify potential peptidase targets of SRP-6, whose antipeptidase activity has been demonstrated for both lysosomal and calpain peptidases and has been implicated in the regulation of necrotic cell death (Luke *et al.*, 2007). It is not well characterized which *C. elegans*-specific proteases may serve as regulatory substrates for SRP-6 in the regulation of necrosis. Therefore, the systematic examination of potential peptidase substrates via RNAi may serve as a useful tool in the identification of genetic SRP-6 targets.

2.2. Identifying serpin targets using biochemistry

While the powerful genetic techniques can provide a great deal of insight into the targets of the *C. elegans*, they do not directly identify a physical interaction. Therefore, a complementary approach is to use affinity purification techniques to coimmunoprecipitate potential targets from transgenic animals expressing a multiple epitope-tagged serpin. This technique, referred to as tandem affinity purification (TAP), was first developed in yeast (Rigaut *et al.*, 1999) and employs the use of two or more affinity tags, separated by a recombinant protease cleavage site fused to the protein of interest at the N- or C-terminus (Table 14.2). This protein can then be purified and concentrated from the complex worm lysate through two rounds of purification to yield the target protein and any potential interactors. This method is particularly useful for low-abundant proteins. Since serpins and the complexed protease are covalently bound (Egelund *et al.*, 1998), the serpin target should be relatively easy to purify. However, this is not usually the case. Two possible reasons have been proposed: (1) serpin: protease complexes contain both inactive protease and serpin and are rapidly turned over (Hirst *et al.*, 2003; Luke, C. J. and Silverman, G. A., unpublished data) and (2) the serpin and enzyme exist under normal conditions in two distinct cellular compartments or cell types and normally only interact under abnormal conditions, for example, SRP-6 and lysosomal cysteine proteases (Luke *et al.*, 2007). However, it is more likely that both hypotheses are true. Although, due to the ease of transgenic animal generation, *C. elegans* is an ideal organism for TAP methods, it has not been widely used to date.

2.2.1. Generation of TAP plasmids for use in *C. elegans*—Several considerations are required when designing a useful vector for TAP techniques: (1) Choice of promoter, (2) Choice of affinity tags, and (3) N- or C-terminal fusions. In general, the native gene promoter is used for TAP plasmids to avoid misexpression in tissues other than the native protein.

This is achieved by amplifying ~2000 bp 5' upstream to the start codon by PCR (discussed in Chapter 13). Although yields of the protein of interest may be lower than if a stronger promoter is used, this may reduce nonspecific interactions generated by overexpression of the gene of interest. The choice of TAP tag is also important. To date, five different TAP tag sequences are being used in *C. elegans* (Table 14.2). Of these, the LAP, SnAvi, and TrAP tags contain GFP, which can be used for both protein purification and protein localization. In general, most TAP tags are placed at the C-terminus of the protein of interest (Cheeseman and Desai, 2005). However, for serpin biochemistry, the C-terminus contains the reactive center loop, which is cleaved by the target protease, releasing a small 3–4 KDa C-terminal fragment. Thus, to purify serpin:protease complexes in context of the *C. elegans* intracellular serpins, the TAP tag is placed at the N-terminus. In our laboratory, we use the TrAP tag. For expression in *C. elegans*, the genomic coding sequence plus the 3' untranslated region of the serpin is amplified by PCR from the genome and cloned in frame downstream of the TrAP tag.

2.2.2. Generation of transgenic lines for purification of serpin targets—Plasmid DNA microinjection is an essential tool for *C. elegans* research and is a straightforward technique for incorporating exogenous DNA into worms (Kimble *et al.*, 1982; Mello and Fire, 1995; Stinchcomb *et al.*, 1985). Transgenic animals are created by microinjection of the plasmid DNA directly into the distal arm of the gonad (Berkowitz *et al.*, 2008). The plasmid DNA encoding a given gene of interest is usually coinjected with another plasmid that serves as a transformation marker, providing either a physical (Kramer *et al.*, 1990) or visual (Gu *et al.*, 1998) phenotype by which to selectively identify transgenic animals. The plasmid DNA undergoes recombination to form extrachromosomal arrays that contain multiple copies of the coinjected DNAs in transgenic animals produced via microinjection (Mello *et al.*, 1991). However, extrachromosomal arrays are not passed on to all progeny. Therefore, to maximize the amount of serpin that can be purified, it is advisable to integrate the array into the genome of the animal. For purification of serpin:protease complexes, it is necessary to create transgenic lines expressing the TrAP tag: serpin fusion protein with the knockout allele, for example, TrAP: :SRP-6 in the *srp-6(ok319)* knockouts. This will then maximize the yield of serpin:protease complexes obtained by eliminating the native serpin that will not be purified by the TAP procedure. The methods for creating transgenic lines and integration of the extrachromosomal array are discussed in detail in Chapter 13.

2.2.3. Purification of serpin:protease complexes from *C. elegans*

2.2.3.1. Large-scale *C. elegans* growth: Once an integrated transgenic line is generated, standard growth procedures will not produce enough worms to isolate sufficient protein for subsequent analysis. Therefore, in our laboratory, we use the following procedures for growing large quantities of worms.

1. The yolks from five large chicken eggs are separated from the egg whites and placed into a 500 ml sterile bottle and mixed with 200 ml of LB.
2. This mixture is incubated at 60 °C for 1 h and then cooled to room temperature rapidly on ice.

3. 20 ml of OP50 (grown up from an overnight culture) is added to media and then 5 ml is placed on top of 100 mm NGM plates.
4. The egg plates are allowed to settle overnight. The remaining liquid was poured off and the plates are dried. Egg plates can be stored at 4 °C for approximately 2–3 weeks until ready for use.
5. Onto 10 egg plates, 75 adult *C. elegans* are placed per plate. Worms are then grown at room temperature for 5–6 days.
6. Once the egg plates are full of gravid adult *C. elegans*, worms are washed off with M9 and pelleted via gravity on ice. Pellets are then frozen at – 80 °C overnight in a 15-ml conical tube.

2.2.3.2. Preparing *C. elegans* lysates: Once enough transgenic worms have been isolated, we use the following protocol for preparing worm lysates:

1. Worms are thawed on ice and resuspended in approximately 4 ml of lysis buffer (50 mM Tris–HCL, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and Complete EDTA-free protease inhibitor cocktail (Roche)).
2. Resuspended worms are passed five times through a French press (Thermo Scientific) at 1000 PSI.
3. The resultant lysate is then centrifuged for 2 min at 700×g at 4 °C to pellet the worm cuticles as well as any worms that have not lysed. This supernatant is placed in new 1.7-ml microcentrifuge tube.
4. The lysate is then centrifuged at 16,000×g for 10 min to remove nuclei and mitochondria. The supernatant is then transferred to a fresh 1.7-ml microcentrifuge tube.
5. The total protein content of the lysate is determined by Bradford Protein Assay (BioRad).
6. Lysates can be immediately used for purification or can be frozen at – 80 °C for several weeks before continuing

2.2.4. Immunoprecipitation—To purify the protein, we employed a novel method in which the protein of interest was tagged with three purification epitopes: two epitopes to aid in purification and a third epitope to identify the protein (TrAP Tag). The first two epitopes were separated by a cleavage site, TEV; the second and third epitopes were separated by the Precision protease site (Table 14.2). All the antibodies used to purify the TrAP: :serpin proteins were affinity-purified, desalted using Zeba Desalting columns (Promega), and coupled to CNBr-activated sepharose 4B (GE healthcare).

2.2.4.1. Coupling antibody to CnBr beads

1. Approximately 500 µl of CnBr sepharose powder is added to 10 ml of sterile PBS in a 15-ml conical tube. Five hundred microliters of dry beads will swell to approximately 1 ml of wet bead volume.

2. The sepharose beads are centrifuged at 500×g for 5 min at 4 °C, then washed three times with 10 ml of sterile PBS.
3. After the final wash, the beads are resuspended in 1 ml of PBS + 0.1% Tween-20 (PBST) to produce an ~50% slurry.
4. Two hundred microliters of 50% bead slurry is transferred to new 1.7-ml microcentrifuge tube.
5. A total of 1 mg of affinity-purified desalted GFP or c-Myc antibody is then added to beads. The binding capacity of the CnBr beads is 6.5– 13 mg/ml of bead volume (or per 2 ml of 50% slurry). However, if total saturation is achieved, this can cause clogging of the beads. The optimal amount of antibody bound should be around 80% of maximal binding.
6. The bead and antibody are nutated for 1 h at 4 °C (or until all the antibody is bound as determined by the lack of protein in the supernatant using the Bradford assay), centrifuged at 500×g in a tabletop microfuge, and the supernatant removed.
7. After the entire antibody has bound, 1 ml of PBST + 0.5 mg/ml BSA is added to beads and then nutated for another hour at 4 °C to block any used binding sites and reduce nonspecific interactions. The beads are again centrifuged at 500×g in a tabletop microfuge and the supernatant removed.
8. Any additional free sites are quenched by the addition of 1 ml of 1 M Tris (pH 7.4) + 0.1% BSA and nutated at 4 °C for 16 h.
9. Beads are then washed three times with 1 ml PBST.
10. Beads were stored as a 50% slurry of PBST + 0.5 mg/ml BSA + 0.01% sodium azide at 4 °C.

2.2.4.2. First and second round of purifications

1. The worm lysate (approximately 2 mg/ml) in lysis buffer (50 mM Tris– HCL, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, and EDTA-free protease inhibitor mixture (Roche)) was precleared by nutating for 2–4 h at 4 °C in the presence of Protein A beads (Immobilized rProtein A IPA 3000, Repligen).
2. The beads are then washed with lysis buffer three times to remove excess sodium azide. Approximately 40 µl of antibody-coupled CnBR beads were then added to the total worm lysate and nutated for 2–4 h at 4 °C.
3. The beads are then centrifuged at 500×g and washed a total of five times with 300 mM NaCl.
4. After these washes, the beads are then blocked with PBS and 5% BSA for 2 h at 4 °C to prevent the TrAP: serpin from nonspecifically sticking to the beads after TEV cleavage.
5. Beads were then resuspended in 30 µl of 300 mM NaCl and incubated with the TEV protease overnight at 4 °C.

6. The supernatant is removed to a new tube, and the beads were washed twice with small volume of 300 mM NaCl. With each wash, the wash was added to the original tube. An equal volume of water was added to the Eppendorf tube bringing the final concentration of the protein in solution to 150 mM to facilitate binding of the beads for the second round of purification.
7. The protein solution is incubated with approximately 80 µl of the second antibody-coupled CnBr beads for 2–3 h at 4 °C.
8. The beads are then washed two times with PBST and resuspended in sample buffer, boiled for 5 min, and centrifuged.
9. Samples were loaded on 10% SDS-polyacrylamide gel (Bio-rad Criterion Tris–HCl) and run at 30 mA before transferring to nitrocellulose by standard Western blotting techniques.

An example of such a Western blot is shown in Fig. 14.2. The blot shows that TrAP: α serpin is immunoprecipitated from the worm lysate using GFP polyclonal antibody-coupled beads, running at an apparent molecular mass of ~75 KDa (the calculated size of TrAP: α serpin; Fig. 14.2, lane 1). Cleavage with the TEV protease results in most of the proteins being released into the supernatant (Fig. 14.2, lane 3). The supernatant from the TEV cleavage is then subjected to further purification using c-Myc epitope tag polyclonal antibody coupled to CnBr beads (Fig. 14.2, lane 4). The Western blot was developed using the FLAG monoclonal antibody, which is part of the TrAP tag. This data demonstrates the functionality of the TrAP tag which should lead to identification of the serpin targets by subsequent mass spectrometry.

Acknowledgments

The authors would like to thank the entire *C. elegans* research community for their continued help and support for over a decade. This work was supported, in whole or in part, by National Institutes of Health Grants DK079806 and DK081422 (to G. A. S.). S. R. B. is supported through grant HD052892 Department of Human Health Services, Public Health Services. M. T. M. is supported by an individual NRSA F32DK086112 from the National Institute of Diabetes and Digestive and Kidney Diseases. C. K. C. is a graduate student, part of the Cell Biology and Molecular Physiology Graduate Program (CBMP) of the University of Pittsburgh and supported through the CBMP teaching fellowship.

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Figure 14.1.
General schematic of the methodologies employed for identifying serpin targets in *C. elegans*.

Figure 14.2.

Western blotting using the FLAG monoclonal antibody of the rounds of purification in the general TAP procedure using the TrAP tag: α -serpin fusion. Lane 1 shows protein eluted after first round of purification with the GFP antibody. Lane 2 shows the amount of TrAP: α -serpin remaining on the beads and lane 3 is the amount of protein in the supernatant after cleavage with TEV. Lane 4 shows the amount of protein eluted after second round of purification with the c-Myc epitope antibody.

Table 14.1
Functional *C. elegans* serpins

<i>C. elegans</i> serpin	<i>In vitro</i> protease targets ^a	Closest human homologue ^b	Knockout allele(s) ^c
SRP-1	Cathepsin K	SERPINA5	ok262
SRP-2	Granzyme B; lysosomal cysteine proteases	SERPINI1	ok350; tm744
SRP-3	Chymotrypsin-like	SERPINB1	ok1433; tm1401
SRP-6	Lysosomal cysteine proteases; calpains	SERPINI2	ok319; tm1994
SRP-7	Unknown	SERPINB4	ok1090

^aReviewed in (Silverman *et al.*, 2010).

^b<http://www.wormbase.org>, release WS219, October 26, 2010.

^c Alleles starting with ok are generated by the *C. elegans* Gene Knockout Consortium (<http://celeganskoconsortium.omrf.org/>); alleles starting with tm are generated by the National Bioresource Project for the Experimental Animal Nematode *C. elegans* (<http://www.shigen.nig.ac.jp/c.elegans/index.jsp>). Allelic information is deposited at <http://www.wormbase.org>, release WS219, October 26, 2010.

Table 14.2
Affinity tags used for tandem affinity purification in *C. elegans*

TAP tag name	Composition	Reference
LAP	HA: :8 × His: :TEV: :c-Myc	Polanowska <i>et al.</i> (2004) ^a
	GFP: :TEV: :S•Tag	Cheeseman and Desai (2005) ^b
	Protein A: :TEV: :CBP	Gottschalk <i>et al.</i> (2005) ^c
SnAvi	SB1: :SB2: :2×TEV: :GFP: :Avi	Schaffer <i>et al.</i> (2010) ^d
TrAP	GFP: :2×TEV: :c-Myc: :Pre: :Flag	Bhatia, S. R. <i>et al.</i> (unpublished)

Abbreviations: HA, Human influenza hemagglutinin tag; His, Histidine tag; TEV, tobacco etch virus protease recognition site; GFP, green fluorescent protein; CBP, calmodulin-binding peptide; SB, SB1 monoclonal antibody epitope; Avi, Avidin; Pre, precession protease recognition site.

^aPolanowska *et al.* (2004).

^bCheeseman and Desai (2005).

^cGottschalk *et al.* (2005).

^dSchaffer *et al.* (2010).