Chapter 2

Methods to Monitor the Functional Subproteomes of SERPIN Protease Inhibitors

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Abstract

Conformational variants of the unique family of protease inhibitors annotated as SERPINs are most often underrepresented in proteomic analyses. This limits understanding the complex regulation that this family of proteins presents to the networks within the protease web of interactions. Using bead-based separation provided by a family of proteomic enrichment products—notably **AlbuVoid™** and **AlbuSorb™**, we demonstrate their utility to satisfy investigations of serum SERPINs. We also suggest their use to develop functional profiles of the SERPIN proteoforms, and how those can establish relationships to disease phenotypes, gene mutations, and dysregulated mechanisms.

Key words SERPIN, SERPIN function, Functional proteomics, SERPIN mechanism, SERPIN biomarkers, SERPIN proteoforms

1 Introduction

The balance and regulation of proteolytic activity within serum is essential to blood based biomarker discovery and possibly to therapeutic intervention. Changes in blood components often reflect acute responses to thwart external stresses, such as coagulation when skin is severed, or inflammatory response during microbial infections. These fast-acting responses are controlled by proteolytic cascades, essentially modifying functionality by the controlled degradation of protein structures. While necessary for acute response, persistent activation of these proteolytic cascades can lead to chronic conditions. So, there is a balance and regulation of these proteolytic cascades which is necessary to keep aberrant proteolysis controlled.

This is done through systemic regulatory protein factors, called protease inhibitors or antiproteases. It is now quite apparent that the influence of inhibition can be just as important as zymogen activation in rapid switch cascades controlling subnetworks within the protease web [1]. One such example of this web's complexity, is

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that one substrate (Neutrophil Elastase) for Alpha-1-Antitrypsin (the inhibitor), can activate the inactive zymogen proMMP-2, a metalloproteinase involved in tumor invasion and angiogenesis [2]. So it becomes necessary to consider that inhibitors are themselves being regulated under different and often complex means of regulation. Within this context, therein lies the special case of the SERPIN superfamily of protease inhibitors.

1.1 The SERPIN Superfamily of Suicidal Inhibitors

The SERPIN family of suicidal serine protease inhibitors plays an integral role in regulating a wide variety of biological activities, and represent 2–10% of circulating plasma proteins. SERPINs regulate coagulation, hormone transport, complement and inflammation, angiogenesis, and blood pressure along with many other pathways. Among the key regulators in blood serum, SERPINA1 (also known as α1-antitrypsin) protects lung tissue from neutrophil elastase, SERPINC1 (also known as antithrombin) controls coagulation proteases, SERPING1 (also known as plasma C1 inhibitor) regulates complement activation, and SERPINF2 (also known as α-2-antiplasmin) inhibits plasmin and regulates fibrinolysis [3, 4].

This unique family of protein inhibitors has been associated with progression or remission of cancer and so they may become valuable biomarkers for therapeutic or diagnostic use. Of clinical utility, prostate-specific antigen (PSA), also known as kallikrein-3, is commonly used as a biomarker for prostate cancer. However, the kallikrein protease family of proteins is of very low abundance in plasma, making observation and quantification difficult. Nevertheless, PSA is regulated by the SERPIN inhibitor family; in men with prostate cancer the ratio of free (unbound) PSA to total PSA is decreased, suggesting a greater role of inhibitory capacity in cancer.

By way of these examples, rather than focusing proteomic discovery efforts on low-abundance proteins like the tissue kallikreins, it may be advantageous to profile much higher abundance Tissue Kallikrein inhibitors like SERPINA5 (Protein C Inhibitor), SERPINA3 (Antichymotrypsin), and SERPINA4 (Kallistatin), to better understand underlying disease mechanisms and potentially generate new biomarkers. However, the role of SERPINs in these critical junctures is rarely straightforward as would be the case for more simplistic binary binding inhibition. For functional interpretation, reliance on strict abundance measurements, such as data that might be derived by ELISA or quantitative LC-MS, does not differentiate the subpopulations of the seemingly opposing outcomes of the SERPIN interaction with its target protease.

This is because SERPINs differ from all other families of protease inhibitors in having a complex mechanism of action that involves a drastic change in their shape, forming the basis of a suicidal substrate inhibition mechanism [3, 4]. The reactive center loop (RCL) extends out from the body of the protein and directs binding to the target protease. The protease cleaves the SERPIN at

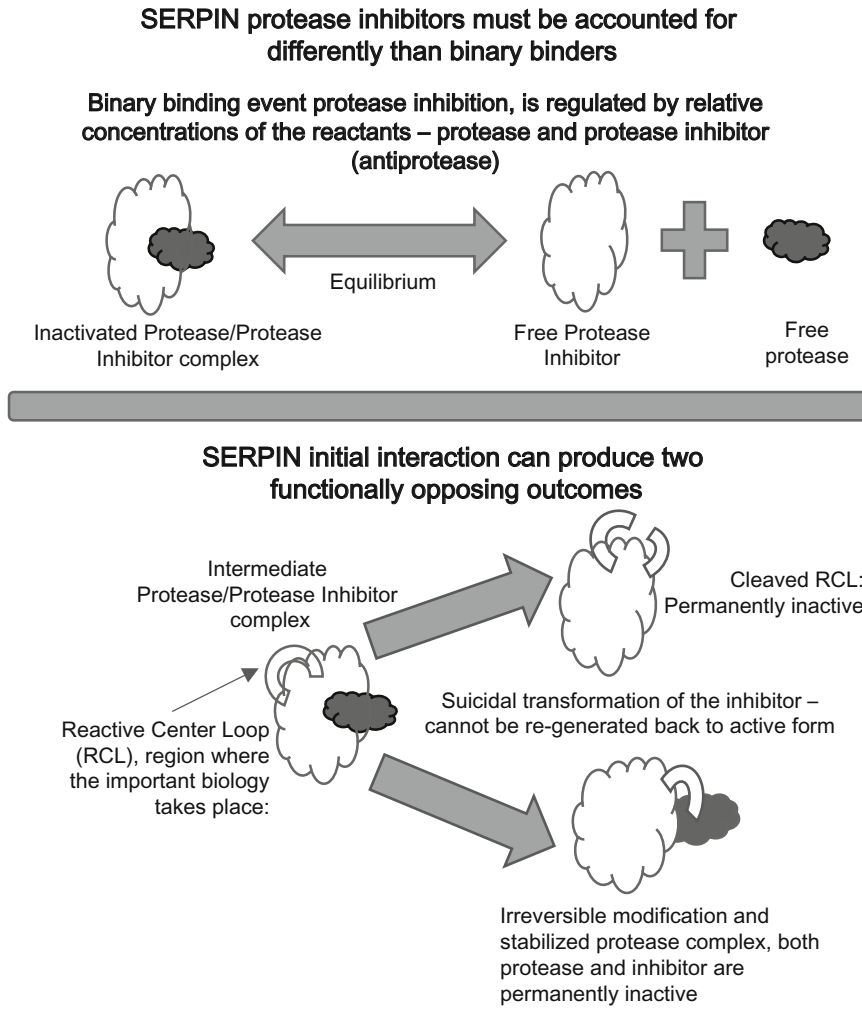


Fig. 1 SERPIN protease inhibitors must be accounted for differently than binary binders

the reactive bond site within the RCL, establishing a covalent linkage between the carboxyl group of the SERPIN reactive site and the serine hydroxyl of the protease [4]. The resulting inactive serpin-protease complex is highly stable, and the structural disorder induces its proteolytic inactivation. As a consequence, the protease is permanently inhibited and functionally inactivated. Nevertheless, the story does not end there for the inhibitor, as after the initial interaction with the substrate protease, one of two possible outcomes can occur, Fig. 1.

One possible outcome is driven by covalent modification permanently inactivating the inhibitory capacity as the SERPIN peptide reactive bond region is irreversibly bound to the protease, and thus cannot be reconstituted back to an active form. The second possible outcome is a permanently inactive variant of the SERPIN

as the peptide RCL region is cleaved and can no longer bind target substrates [4]. As a result, even minor changes in the structure due to genetic variation and posttranslational modifications can modify the function of SERPINs and give rise to a variety of clinical presentations. Some 200 different mutations in serpins are known to result in disease [5]. In particular, mutations affecting antithrombin confer a predisposition to thrombosis, those affecting C1 Inhibitor confer a predisposition to angioedema, and those affecting antiplasmin confer a predisposition to hemorrhage. Interestingly, an alternative function is made possible by a mutation in which the methionine in the RCL region of Alpha-1-Antitrypsin is replaced by an arginine converting its function as an inhibitor of neutrophil elastase to a highly effective inhibitor of the coagulation proteases, the consequence of which is life-threatening hemorrhagic disease [6].

Mutations can affect function throughout the sequence. However, the most common loss of serpin function from mutation are those affecting the mobile hinges of the molecule within or near the RCL. These lead to spontaneous changes in conformation that allow either the insertion of the intact reactive loop into the main β sheet, resulting in the formation of an inactive "latent" form, or the insertion of the loop of one molecule into the β sheet of the next, resulting in the formation of polymers. Polymerization occurs in Alpha-1-Antitrypsin with the common Z variant mutation, leading to decreased secretion from the liver into the circulation, resulting in emphysema and cirrhosis [7]. Amino acid substitution in the RCL region is the likely event transforming the non-inhibitory serpins. Posttranslational modifications at the RCL region such as oxidation of methionine in Alpha-1-Antitrypsin have also been proposed as a source of dysfunction [8].

So understanding the underlying mechanisms, contributions from genetic wiring or environmental stresses, and their relationships with aberrant proteolysis is necessary to characterize disease. Functional proteomic analyses offer a new lens of observation to examine the resulting conformational variants that can be reported as potential biomarkers of disease phenotypes. As an example, one such inhibitor SERPINA1, known more commonly as Alpha-1-Antitrypsin (AAT), has several isoforms observed in plasma using 2-DE, and often serves as a model for conformational diseases [5, 9]. Circulating levels of AAT are between 1.2 and 2 mg/mL in healthy persons, but are known to increase during acute phases of inflammation and infection. Its function and activity is controlled by the many variants attributable to its conformational nexus of features; the term "proteoform" is often used to describe such conformational variability and we adopt that term here.

Other reports observe that the conformational properties of AAT have multiple effects on tumor cell viability and diverse roles in tumorigenesis, suggesting such isoforms may display a specific basis

for diagnosis of cancer and neurodegenerative disorders [8, 10, 11]. Yet, most often in proteomics, all subpopulations of SERPINs are rolled into and counted as one homogeneous population. As a result, the regulation, balance and dynamism within these systems and its impact on the protease web of disease progression cannot be properly investigated, and indeed conclusions based on such measurements may be very misleading.

So methods that account for important distinctions among the many subpopulations generated by conformational variants within this superfamily of proteins are considered in this chapter. Specifically, a functional proteomic investigation of the two seemingly opposing outcomes of the initial inhibitor-protease interaction can be monitored:

1. The total amount of potentially inhibitory SERPIN activity as reported by an intact RCL region.
2. A transformed subpopulation of the inhibitor, as reported by a cleaved RCL region permanently inactivating its inhibitory potential.

1.2 New Methods to Functionally Profile SERPINs

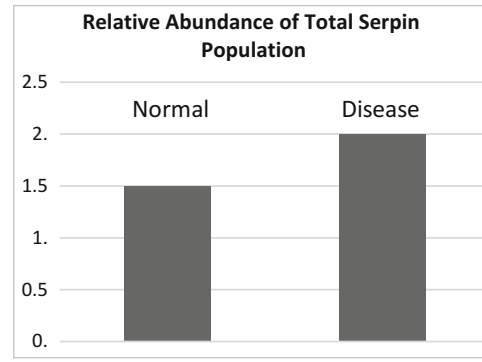
By combining unique strategies of binding and voiding high-abundance proteins, we can observe different subpopulations with characteristic binding biases. We have previously reported for Alpha-1-Antitrypsin that the resultant cleaved-RCL proteoform and the uncleaved-RCL proteoform are very distinctive subpopulations, separated by **AlbuVoid™**, and reported at the peptide feature level by LC-MS [12]. In this chapter, we consider how Albumin Removal products—**AlbuVoid™** and **AlbuSorb™** (Biotech Support Group LLC, Monmouth Junction, NJ, USA), can help to functionally profile and unravel this complex biology of the SERPIN superfamily of proteins.

Through a proprietary polymer coating, 50 μm porous silica beads are crosslinked and passivated. This is the foundation of the **NuGel™** surface chemistry. Mixed-mode of binding interactions form the basis of general nonspecific protein adsorbents or beads with weak affinity or imperfect fit interactions. In this way, binding behavior is very different from classical high affinity binding which demands near perfect fits. Under protein saturation conditions, progressive displacement provides a separation bias towards or against select proteins. As a result, all derivative **NuGel™** products were empirically characterized to meet the needs of the application, for example, **AlbuVoid™** to selectively void (not bind) Albumin with special bias toward the vast majority of the remaining low-abundance serum proteome on the bead. Two **NuGel™** based products support Albumin Removal:

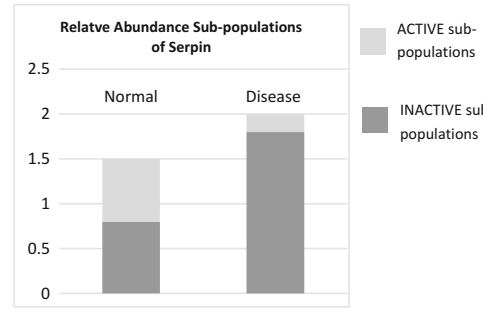
1. **AlbuSorb™** and **AlbuSorb™ PLUS** (also binds immunoglobulins) for selective binding of Albumin.

2. **AlbuVoid™** for negative selection or voidance of Albumin with consequent enrichment of the remaining serum sub-proteome on the bead.

So, while other proteomic methods might observe this:
Past observations:



We describe methods to observe this:



In this hypothetical case, the ratio of the ACTIVE subpopulation vs. the INACTIVE subpopulation is greatly altered in disease, whereas simple abundance measurements of the total population would not be very informative (*see* **Notes 1** and **2**). In the following Table 1, we report on the SERPINs observable by LC-MS and how they bias toward **AlbuVoid™** and **AlbuSorb™**, as measured by spectral counting.

We suspect that conformational changes associated with the cleavage of the reactive bond confer more or less binding affinity to the nonspecific interactions with our beads. Such cleavage stabilizes the SERPIN structures; **AlbuVoid™** binding especially biases toward unstructured proteins, and we have previously reported the SERPINA1 (Alpha-1-Antitrypsin) RCL-intact proteoform binding favorably over the RCL-cleaved proteoform [12]. Noteworthy is that several non-inhibitory SERPINs A6-8, all bind poorly to **AlbuVoid™**, supporting evidence for the role of conformational stability in binding biases.

Table 1
Serum SERPINs observable by LC-MS

Protein ID	Also known as (conc.)	Function	AlbuVoid™ bead bound S.Cts.	AlbuSorb™ flowthrough (unbound) S.Cts.	Reactive (RCL) bond site	Notable variants
SERPINA1	Alpha-1-Antitrypsin (AAT) (1–2 mg/mL)	Inflammation, elastase inhibition	59 (strong bias toward RCL-intact proteoform)	519	Met382-Ser383	Z variant {Glu366 → Lys366} deficiency syndrome, Pittsburgh variant {Met382 → Arg382} life-threatening bleeding
SERPING1	Plasma protease C1 inhibitor (0.25 mg/mL)	Regulates complement cascade, levels rise ~2-fold during inflammation	51	63	Ala465-Arg466 chymotrypsin, Arg466-Thr467	
SERPINA3	Antichymotrypsin (100–500 μg/mL)	Apoptosis, Alzheimers, inflammation	86	117	Leu383-Ser384	
SERPIND1	Heparin cofactor II (40–80 μg/mL)	Coagulation, thrombin inhibitor activated by heparin	124	28	Leu463-Ser464	
SERPINA8	Angiotensinogen (AGT) (40–60 μg/mL)	Angiotensin I precurser, blood pressure regulation, non-inhibitory	4	62	None	Disulfide bond is labile, near 40:60 ratio with the oxidized disulfide-bonded form
SERPINC1	Antithrombin, ATIII (0.12 mg/mL)	Inhibits thrombin, regulates coagulation, angiogenesis, heparin cofactor	58	79	Arg425-Ser426	Mutations/variants can lead to increased risk of thrombosis, alter functional heparin and thrombin binding domains

(continued)

Table 1
(continued)

Protein ID	Also known as (conc.)	Function	AlbuVoid™ bead bound S.Cts.	AlbuSorb™ flowthrough (unbound) S.Cts.	Reactive (RCL) bond site	Notable variants
SERPINF1	Pigment epithelium-derived factor, PEDF (20–175 μg/mL)	Neurotrophic factor, non-inhibitory	45	0	None	
SERPINA4	Kallistatin (20 μg/mL)	Kidney function, inflammation	45	0	Phe388-Ser389	Cleavage at the reactive site by tissue kallikreins
SERPINF2	α-2-antiplasmin (60–80 μg/mL)	Fibrinolysis, inhibitor of plasmin and trypsin	10	39	Arg403-Met404 plasmin, Met404-Ser405 chymotrypsin	Alanine insertion at the reactive site promotes serious bleeding disorders
SERPIN-A10	Z-dependent proteinase inhibitor (1–2 μg/mL)	Coagulation regulation	23	0	Tyr408-Ser409	Tyr408 → Ala408 loss of inhibition
SERPINA5	Protein C inhibitor (5 μg/mL)	Coagulation, inflammation	13	0	Arg373-Ser374	Variants near or at the reactive bond alter inhibition of thrombin activity
SERPINA6	Corticosteroid-binding globulin (60–80 μg/mL)	Hormone transport, non-inhibitory	0	26	None	
SERPINA7	Thyroxine-binding globulin (15 μg/mL)	Hormone transport, non-inhibitory	0	17	None	

2 Materials

Items required	Reagent
AlbuVoid™ beads	Manufacturer supplied
Binding buffer AVBB, *PH 6.0*	Manufacturer supplied
Wash buffer AVWB, *PH 7.0*	Manufacturer supplied
SpinX centrifuge tube filters	Manufacturer supplied
Trypsin, DTT, iodoacetamide	Not supplied

3 Methods

For this chapter, we shall consider only the workflow supporting **AlbuVoid™**, but LC-MS workflows supporting **AlbuSorb™** would be similar, taking into account which fractions would contain the majority of Albumin and which do not, Fig. 2.

The workflow follows the **AlbuVoid™ LC-MS On-Bead** sample prep method following the manufacturer's protocol. In brief, 50 μL serum is prepared by adding a binding buffer, then applied to the **AlbuVoid™** beads, and washed. All steps are performed within a microfuge spin-filter format. Albumin is most especially voided out, while the majority of the remaining serum proteome is retained on the bead. After the final wash, reduction, alkylation, and Trypsin digestion all take place on the bead.

For best results—the serum should be clear and free of colloidal material. We recommend first filtering through a 0.45 μm syringe-type filter before beginning the prep.

In bold are the **AlbuVoid™***LC − MSOn − Beadkitcomponents*.

1. Weigh out 25 mg of **AlbuVoid™** bead in a spin-tube (**0.45 μ SpinX centrifuge tube filter supplied**).
2. Add 125 μL of **Binding Buffer AVBB**. Vortex for 5 min at room temperature followed by centrifugation at 1500 × *g*. Discard the supernatant.
3. Repeat **step 2**.
4. Condition clarified serum by adding 100 μL of **AVBB** to 50–100 μL of the Serum. Using a syringe-type micro-filter, clarify the serum. Add sample to the **AlbuVoid™ beads** in **step 3**. Vortex for 10 min and then centrifuge for 5 min at 10,000 × *g*.
5. Discard the albumin filtrate.
6. To the beads, add 250 μL of **Wash Buffer AVWB**. Vortex for 5 min and centrifuge for 4 min at 10,000 × *g*. Discard the Wash.

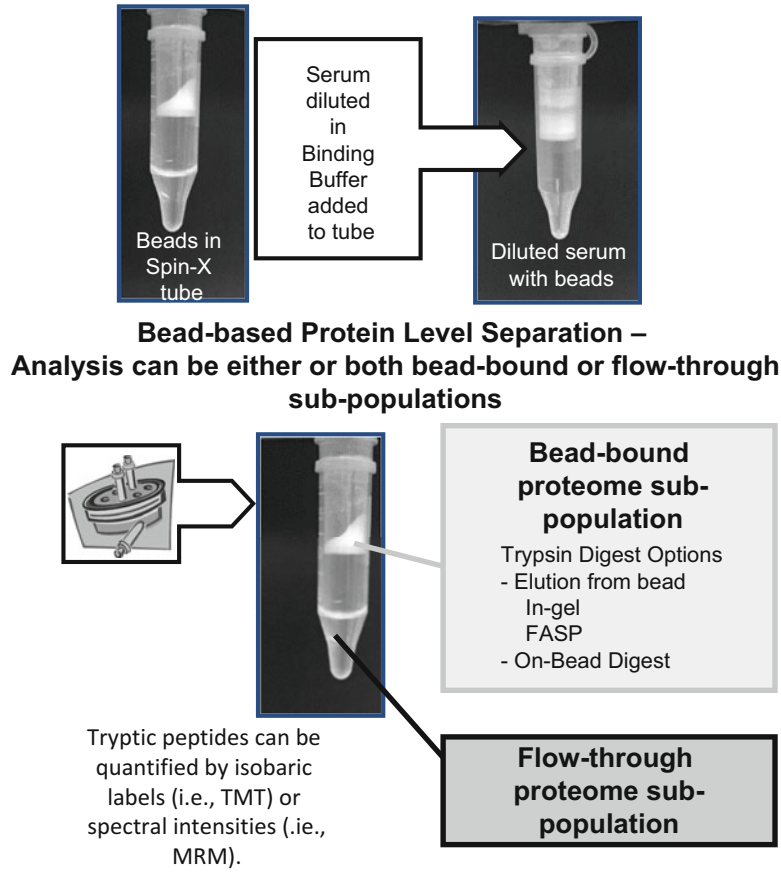


Fig. 2 Enrichment/depletion option for serum proteome separations

7. Repeat **step 6** two times.

   The AlbuVoid™ beads are now enriched with albumin-depleted low-abundance proteins. For LC-MS sample preparation, the on-bead digestion protocol is as follows. Option—the proteins can be eluted with 0.25 M Tris, 0.5 M NaCl, pH 10 (*see* **Note 3**).
8. After the final wash steps from **step 7** from the enrichment, add 10 μL 100 mM DTT + 90 μL **Wash Buffer AVWB**, vortex 10 min, incubate ½ h at 60 °C.
9. After cooling, add 20 μL 200 mM Iodoacetamide and 80 μL **Wash Buffer AVWB**, incubate in dark for 45 min at room temp.
10. Centrifuge at 10,000 × *g* (microfuge max setting) for 5 min, and discard supernatant.
11. Add 40 μL sequencing-grade trypsin (0.4 μg/μL, in 50 mM acetic acid) + 60 μL **Wash Buffer AVWB** to the beads. Digest

overnight (maximum) at 37 °C or other suitable time period (s).

12. Centrifuge at 10,000 × *g* (microfuge max setting) for 5 min and retain peptide filtrate.
13. To further extract remaining peptides, add 150 μL 10% formic acid, vortex 10 min, centrifuge at 10,000 × *g* (microfuge max setting) for 5 min, and add this volume to the first volume.
14. Total is about 250 μL. Prepare to desired final concentration. Store at −80 °C until LC-MS/MS.

Example of LC-MS reporting features.

After TMT labels (Proteome Sciences plc, Surrey, UK) labeling, the peptides are pooled and analyzed with a single LC-MS/MS 3 h gradient run using nanoRSLC system interfaced with a Thermo Scientific™ Q Exactive™ HF (Thermo Scientific) instrument, using data-dependent acquisition with resolution of 60,000, followed by MSMS scans (HCD 30% of collision energy) of 20 most intense ions, with a repeat count of two and dynamic exclusion duration of 60 s (Table 2).

The amino acid region of the RCL is 368–392, so the adjacent RCL tryptic peptide at Lys367, highlighted in gray, serves as a good comparison between the observable serum subpopulations, Fig. 3.

Bead Bound—The subpopulation of proteins that bind and are observed by the **AlbuVoid™** *methods.*

Flow-through (unbound)—The subpopulation of proteins that flow-through the **AlbuVoid™** *beads, unbound.*

Table 2
SERPINA1 (AAT) TMT ratio: pooled pancreatic cancer/pooled normal

		Sp Ct = peptide spectral counts							
				Bead bound		**Flow-through (unbound)**		**Serum untreated**	
SERPINA1 (AAT) Peptide region	**Start**	**Amino acid sequence**	**End**	**TMT ratio**	**Sp Ct**	**TMT ratio**	**Sp Ct**	**TMT ratio**	**Sp Ct**
Adjacent RCL Tryptic	360	AVLTIDEK	367	0.35	9	1.78	21	1.53	14
RCL cleaved	368	GTEAAGAMFLEAIPM	382			1.05	7	1.16	23
RCL intact	368	GTEAAGAMFLEAIPM SIPPEVK	389	0.77	5	1.75	1	1.34	50
RCL cleaved	383	SIPPEVK	389			1.45	27	1.44	18
Total all peptide features				0.54	132	1.57	372	1.44	460

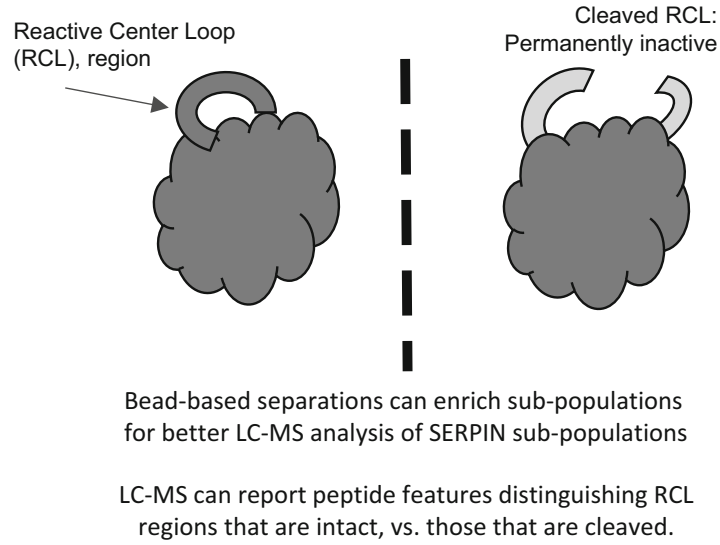


Fig. 3 SERPIN LC-MS reporting features

Untreated—The total population of proteins that are observable in serum without any sample enrichment, that is without the use of **AlbuVoid™**.

Highlighted in dark gray is the RCL intact peptide. Highlighted in light gray are the two RCL peptides that are cleaved at Met382, during suicidal substrate interaction; note that these peptides were not observed in the Bead-Bound fraction. These data suggest that the overall SERPINA (AAT) population is dominated by the subpopulation *up*-regulated and collected in the Flow-through fraction of **AlbuVoid™**, and this same subpopulation dominates the analysis when untreated sera is investigated. Such would be the case in acute AAT up-regulation commonly observed with malignancies and inflammation. However, using our methods we distinguish a subpopulation enriched by the bead and reporting with the bound fraction, as being severely *down*-regulated with cancer! While this observation may have potential biological significance, no conclusion about the particular cancer-specific proteoform uncovered can be made at this time (*see* **Note 4**). Nevertheless, from a biomarker perspective, this serves an additional multiplier benefit; that is the ratio of the two subpopulations report Adjacent RCL Tryptic peptide region as unbound/bound $(1.78/0.35) = 5$. As isobaric label ratios in discovery methods can sometimes compress the reporting difference, this ratio may become much greater once more targeted quantitative methods are developed, a prospect for future tests.

4 Notes

- Bead-based proteomic enrichment methods as described can support the functional and structural proteomic analyses necessary to characterize these conformational subpopulations so that they may become useful biomarkers for disease. It should nonetheless be recognized that the RCL reporting methods described here only work for RCL regions where the cleavage site is *non-Tryptic*, and these must be entered as special peptides into LC-MS computational workflows. "neXtProt: a knowledge platform for human proteins," provides a useful web-based resource for annotating RCL cleavage sites [13]. To distinguish RCL regions where the cleavage site is Tryptic, it becomes necessary to differentiate those sites that are cleaved in vivo by those cleaved ex vivo. Several methods have been developed for this purpose and generally fall under methods called N-terminomics [14]. This is an area for future investigations.
- Classical high-abundance proteins like the SERPIN superfamily (i.e., Alpha-1-Antitrypsin) are often overlooked as potential biomarkers of disease. Yet discoveries certainly can rest in the data-rich features of the diverse variety of conformational and proteoform variants associated with many of the classical serum proteins. When considering these mid- to high-abundance proteins, disease differentiation can be obtained through the discreet quantification of the multiple subpopulations available to measure. The methods described in this chapter can begin to unravel and sort these variant subpopulations so that LC-MS peptide reporting features, and potentially other functional reporting features (i.e., substrate turnover), can distinguish these proteoforms with more functional details. It is our intention that these methods will lead to characteristic disease profiles, which can then be compared and evaluated for eventual biomarker utility.
- Many trypsin digestion protocols have been developed to improve the reproducibility and, in some cases, reduce the digestion time necessary for LC-MS analysis [15]. While we have shown methods that adapt **AlbuVoid™** for on-bead digestion, the bead-based enrichments described here are nonetheless compatible, after elution from the beads, with other common digestion methods, such as filter-aided (FASP) solution methods and post-electrophoresis, in-gel methods.
- By using the peptide reporting features of the RCL peptide regions within SERPIN inhibitors, both "potentially active" and "permanently inactive" proteoforms are now distinguishable, adding a new level of proteomic characterization to the underlying mechanisms of disease. As one example, hereditary

dysfunction of SERPINA1 (Alpha-1-Antitrypsin) has been previously determined as a risk factor for cancer [16]. As many proteins within the SERPIN family proteins are of moderate- to high-abundance quantities in serum (10 + μg range/mL), depleted functionality would impose severe dysregulation to a normal and healthy individual. Several of the key regulators in the Coagulation Pathway such as SERPINA10 (Z-dependent Proteinase Inhibitor) and SERPINA5 (Protein C Inhibitor) have notable genomic variants that alter their inhibitory function [13]. These might therefore be risk factors for disease. So using the methods described in this chapter, hereditary genomic factors that associate with SERPIN function can be further investigated.

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