

Thrombin induces broad spectrum proteolysis in human serum samples

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Abstract

Background: During clotting, α thrombin cleaves fibrinogen releasing fibrinopeptide A (FPA). FPA is easily identified in serum using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). Using MALDI-TOF MS, we observed multiple, progressively shorter fragments of serum FPA. Following ambient incubation of serum, variations in the content of FPA fragments occur over time. Denaturation of α thrombin by heating the serum sample appears to minimize this variation. These observations suggest that intrinsic proteolytic and peptidolytic activity is elevated in serum and perhaps originates from the coagulation cascade enzymes themselves, especially α thrombin.

Methods: Extrinsic addition of α thrombin to a subset (3–30 kDa) of plasma proteins was carried out to induce proteolysis and to examine the resultant peptides to reveal α thrombin susceptible parent proteins. One of these identified proteins, hemopexin, was directly digested by α thrombin and the peptides examined to confirm the observations from the initial plasma protein digestion.

Results: Extrinsic addition of α thrombin to a subset (3–30 kDa) of plasma proteins results in wide-spread digestion of proteins unrelated to coagulation, revealing a substrate range encompassing more than fibrinogen. Direct digestion of one of these proteins, hemopexin, by α thrombin confirms these observations.

Conclusions: The resulting peptides indicate broad tolerance beyond the consensus R-G cleavage site of fibrinogen; in fact, there appears to be no bias for the amino acid following the R/K residue. These data support our hypothesis that the enzymatic activities inherent to coagulation, or at least to thrombin, contribute to destabilization of the protein and peptide content of serum.

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Introduction

Blood is a key tissue that provides a wide array of functions interacting with or perfusing every other tissue or organ in the body (1, 2) and hence it has a central role in diagnostics and research. Serum is a fluid derivative of blood resulting from a proteolytic clotting process followed by centrifugation to remove solid (clot) materials, including cells and other poorly soluble content. Plasma is the liquid portion of blood from which the cells are removed (typically via centrifugation), with blood collection generally requiring chemical inhibition (e.g., via anti-coagulants) of the clotting process. Both serum and plasma can be used for biomarker discovery; however, serum is widely used as a starting material for many diagnostic assays (2). Many current diagnostics rely on the measurement of highly characterized, stable protein or peptide biomarkers. In a number of current studies, blood is expected to be an enormous resource of new protein or peptide biomarkers, but many of these are expected to be either potentially labile or in low abundance, complicated by the tremendous dynamic range of blood protein concentration masking this valuable, dilute content (1, 3, 4).

Coagulation or clotting is a proteolytically driven process, involving a cascade of enzymes including serine proteases, which lead to the formation of "solid" fibrin/platelet plugs (2, 5). The protagonist coagulation enzyme, thrombin (EC 3.4.21.5), is a serine protease with trypsin-like specificity. It cleaves the NH_2 terminal "caps" of chains $\text{A}\alpha$ and $\text{B}\beta$ of fibrinogen, releasing fibrinopeptide A (FPA) and fibrinopeptide B (FPB), and rapidly converting fibrinogen to insoluble fibrin (6, 7).

From a biochemical perspective, the clotting process itself seemingly has the potential to reduce the suitability of serum for proteome analysis. We hypothesize that coagulation not only has the intended outcome of clot formation, but also has the potential ability to degrade many of the other proteins in the blood sample. In terms of "biomarker discovery", this potential proteolysis could especially affect low abundance proteins, and, in fact, could be an underlying cause for poor research outcomes due to proteolytic destruction of potential protein and peptide biomarkers. Thrombin cleavage of the $\text{A}\alpha$ chain of fibrinogen should result in release of the "cap" peptide, known as FPA [FPA, (MH⁺) of 1536.7 Da in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)]. However, a series of truncated FPA, peptides are observed in serum (that are not present in detectable levels in plasma), suggesting abundant exopeptidase activity in serum.

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Compared to plasma, the time from serum sample collection to analysis is extended due to the time needed for complete clotting (minimum 30 min) prior to centrifugation (approximately 10 min). Thus, the inherent nature of the sample prevents a true "time zero" representation of blood proteins. This extended time frame would further amplify any proteolytic damage driven by coagulation. The less like the "in vivo" state a sample becomes, the more difficult the discovery of new biomarkers will be.

There are many examples of the effects of so-called "preanalytical variables" (time, sample variables, collection variables, etc.) on analytical outcome, particularly for protein and proteomic studies (3, 8–11). A recent perspective on the subject has been put forward with specific focus on proteome analyses of blood (12). Variations in cytokine levels within serum vs. plasma have also been demonstrated (13). The variability of the relative peptide intensities in serum, under various conditions, has been observed using MS, including attempts to harness alternative proteolytically derived patterns of common peptides for diagnostic purposes (3, 14, 15). This phenomenon, that extrinsic fibrinopeptides can be degraded by intrinsic enzymes within serum, was reported more than three decades ago (6). The common theme in these works is that control of potential variability requires painstaking care, if it is controllable at all. More recently suggested is the inherent need for sample stabilization by reducing intrinsic proteolytic activity in plasma with protease inhibitors, potentially enabling better detection of labile endogenous peptides, such as bradykinin (*m/z* 1060) (16).

The study we present here demonstrates two distinct enzymatic activities in serum that alter the peptide and protein content of blood after collection. First, we observe a significant amount of peptidolytic activity in serum, evidenced by variations in the observable peptides (using MALDI-TOF MS), as a function of time following serum preparation. Second, we demonstrate a broad *ex vivo* proteolytic potential of α thrombin, extending beyond its presumed high "clinical" specificity for fibrinogen cleavage. Taken together, these data demonstrate extensive direct and/or indirect thrombin-induced proteolysis of the protein and peptide content of serum.

Materials and methods

Human α thrombin (EC 3.4.21.5) was purchased from Enzyme Research Labs (South Bend, IN, USA). 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES minimum 99%), Trypsin (EC 3.4.21.4, sequencing grade), and Tri-FluoroAcetic acid (TFA minimum 99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium hydroxide and ammonium bicarbonate were from Sigma Chemical and α -cyano-4-hydroxycinnamic acid (HCCA) was from Bruker Daltonics (Billerica, MA, USA). Reagent grade water (≥ 18.2 m Ω) was produced in-house.

Hemopexin, purified (> 40%, minimum) from human plasma, was purchased from US Biological (Swampscott, MA, USA).

Blood collection

Blood was collected into serum [BD SST™, Becton Dickinson (Franklin Lakes, NJ, USA) part number 367988] or EDTA plasma (BD Vacutainer® part number 367525, K2EDTA) tubes, using a Safety-Lok™ blood collection set (BD part number 367281) from five healthy subjects. Serum was allowed to clot for 30–40 min at room temperature and then centrifuged at 2500 $\times g$ for 10 min (at room temperature). Plasma was centrifuged (as above) within 10 min of collection. Serum samples were aliquoted immediately following centrifugation into fractions and handled as described below. Plasma was aliquoted directly following centrifugation and either frozen (–80°C) or used immediately.

Sample preparation

Aliquots were placed on ice. Samples designated for thermal inactivation were placed at 65°C for 30 min in a heat block. Following heating, samples were returned to ice to equilibrate. Time 0 aliquots were removed and quenched with TFA to a final concentration of 0.1% (v/v) (to reduce intrinsic proteolysis by acidification) and frozen (–20°C). For the remaining time points, aliquots were removed at specified time intervals and quenched as described above.

Peptides were removed from the quenched samples by ultrafiltration through a Microcon YM10 (10K MW cut-off filter, Millipore, Waltham, MA, USA) according to the manufacturer's instructions. Collected peptides were cleaned and processed for MALDI-TOF MS analysis using Zip-Tips C18 (Millipore) according to the manufacturer's instructions.

A saturated solution (~ 10 mg/mL) of HCCA was prepared (in 50% v/v acetonitrile with 0.1% v/v TFA) for the matrix and 1–2 μ L was mixed with an equal volume of peptide sample and spotted on a stainless steel MALDI target (Bruker Daltonics) and air dried.

Sample analysis

For peptide analysis of serum samples using MALDI-TOF MS, samples (spotted $n=3$) were analyzed using an Ultraflex II MALDI-TOF MS instrument from Bruker Daltonics. Operating in reflectron, positive mode, the MS spectra of peptides between 750 and 4000 *m/z* were collected in an automated mode. To enable a qualitative comparison of samples, the spectra were collected from each sample (total 3000 laser shots) from 30 qualified spectra per sample spot (each 100 shots). Spectra were routinely calibrated against an external control peptide mixture after every 4–6 samples. In our processes, peak intensity within such spectra typically has a coefficient of variation <20% (data not shown). Spectra were normalized (where indicated) against an internal peak (*m/z* 1465.9 – *des* AlaFPA) which has been shown to have superior ionization properties in MALDI MS (17), allowing a more accurate evaluation of various experimental conditions.

Spectra were processed by FlexAnalysis ver. 2.4 (Bruker Daltonics) and reviewed manually. Several peptides were chosen for sequencing by MALDI-TOF/TOF. Spectra were processed by FlexAnalysis and peak lists were analyzed using BioTools (Bruker Daltonics) to determine peptide sequences.

For all TOF/TOF experiments, peptides (and parent proteins) were identified by searching against the NCBI Inr database using Mascot (Matrix Science, Boston, MA, USA). Liquid chromatography (LC)-MALDI was performed using an Agilent nano HPLC 1100 with chemstation software (Agilent Technologies, Palo Alto, CA, USA), coupled to a Proteiner FC (Bruker Daltonics) for MALDI spot deposition. LC-MALDI

MS data collection was performed using WARP-LC software as described below.

Proteolytic activity assay of α thrombin

Low molecular weight (LMW) proteins ($3 > 30$ kDa) were collected from plasma (effectively removing fibrinogen to prevent clotting) by ultrafiltration, by passing the plasma first through a 30-kDa filter (Microcon YM30) and then running the effluent through a 3-kDa filter (Microcon YM3). The retained fraction from the second filter was adjusted to pH 7.4 with HEPES/NH₃OH. Proteolytic digestion was carried out by adding α thrombin (10 U/mL final, approximately 1–2 \times the level observed within a clot activated tube) or trypsin (20 U final), and samples were incubated at room temperature or 37°C. Sample analysis by LC-MALDI was conducted as follows.

The resulting peptides were separated with an Agilent 1200 nano-HPLC using a Zorbax 300SB-C18 (5 μ m), 5 mm \times 0.3 mm trapping column (Agilent Technologies, Santa Clara, CA, USA) followed by a Chromolith™ CapRod™ RP-18 endcapped column (Merck, Darmstadt, Germany) with dimensions of 150 mm \times 0.1 mm for separation. A gradient elution was performed using 0.1% TFA in water (mobile phase A) and 0.1% TFA in acetonitrile (mobile phase B) at 2 μ L/min. The gradient used was as follows: hold B at 2% for 7 min, then increase from 2% to 30% B over 30 min, then increase B to 90% over 20 min. Eluted peptides were monitored at 214 nm using an 80-nL flow cell.

Fractions were spotted onto a 400- μ m AnchorChip™ (Bruker Daltonics) every 15 s using the Proteiner FC™ (Bruker Daltonics). Warp-LC™ software (Bruker Daltonics) was used to automate the MS collection process, using MS methods described above, and to create a peak list for MS/MS analysis based on a signal/noise threshold of greater than 20, and data were analyzed as above.

Partially purified hemopexin (>50% purity from human plasma) was purchased from US Biological. This sample was heat-treated (65°C for 30 min) to eliminate any contaminating proteolytic activity that might be present from this partially pure starting material, and peptides were removed by ultrafiltration (YM10 cutoff filter). The absence of peptides and intrinsic proteolytic activity in the resulting hemopexin preparation was demonstrated using MALDI MS, monitoring both immediately and over time. Dilutions of prepared hemopexin [2, 20, and 200 μ g/mL in phosphate buffered saline (PBS) pH 7.4] were digested with α thrombin (0.1, 1,

and 10 U each) for 16 h at 25°C. The same dilutions of hemopexin were digested with trypsin (1 μ g) as a positive control.

Results

FPA peptides in serum vs. plasma

Thrombin cleaves fibrinogen to initiate clot formation and results in release of soluble FP fragments. The full-length FPA has the sequence (ADSGEGDFLAEGGGVR) and a MALDI [MH⁺] mass-to-charge ratio (m/z) of 1536.94 (Table 1). This peptide also exists in a phospho-serine form, FPA-P (ADP-SGEGDFLAEGGGVR) resulting in an m/z of 1616.94. Serum and plasma samples from five subjects were analyzed for the presence of FPA peptides.

A series of progressively shorter peptides, sequentially truncated from the amino terminus, are easily observed in serum with direct MALDI analysis (Table 1). We were unable to observe similar peptides in plasma (EDTA anti-coagulant) with direct MALDI. In case ion suppression may have reduced or impeded the detection of these truncated FPs in plasma, LC-MALDI was employed to provide improved sensitivity and a more consistent view of peptide content. In general, LC-MALDI enabled the detection of more peptides, including very small amounts of the shorter FPA observed in serum. Table 1 displays the FPA peaks identified as well as their average peak intensities in both serum (direct MALDI) and plasma (LC-MALDI). The expected dramatic difference in serum (high) vs. plasma (low) FPA concentration is evident, since FPA is essentially a by-product of clot formation. Direct MALDI analysis of serum revealed ten easily identified FPA fragments. Peak intensity comparison between plasma and serum is qualitative as different methods were required to visualize them; however, within plasma, essentially only the direct thrombin cleavage product (m/z 1536.9) as well as the singly-truncated *des* Ala peptide are obvious (refer to Table 1 for sequence), while two additional peptides (m/z 1077.6; 1206.7) were detected at much lower intensity (Table 1). Peptide sequences from both direct MALDI

Table 1 FPA and FPA-derived peptides observed in serum.

m/z	Length	Sequence	PTM	Plasma ^a	Serum
905.6	9	FLAEGGGVR		ND	20,240
1020.6	10	DFLAEGGGVR		ND	31,465
1077.6	11	GDFLAEGGGVR		40	34,882
1206.7	12	EGDFLAEGGGVR		80	166,824
1263.7	13	GEGDFLAEGGGVR		ND	65,365
1350.8	14	SGEGDFLAEGGGVR		ND	94,222
1465.8	15	DSGEGDFLAEGGGVR		1400	235,010
1536.9	16	ADSGEGDFLAEGGGVR		900	18,297
1545.8	15	DSGEGDFLAEGGGVR	phospho-S	ND	20,395
1616.9	16	ADSGEGDFLAEGGGVR	phospho-S	ND	30,400

Thrombin cleaves fibrinogen yielding peptide m/z 1536.9 as the primary (full-length) cleavage product. Shorter fragments, successively truncated from the amino terminus, are observed in serum, along with serine phosphorylation variants. TOF/TOF MS confirms peptide sequences (data not shown). Comparison of FPA peptide intensities observed in plasma (EDTA) and serum using MALDI MS are listed in the appropriate columns. ND indicates peptides that were not observed in plasma, even after more sensitive LC-MALDI analysis. ^aDue to low intensities direct MALDI was not adequate for FPA peptide analysis in plasma, therefore LC-MALDI was employed to visualize FPA peptides. PTM, post-translational modification.

and LC-MALDI experiments were confirmed using TOF/TOF MS sequencing analysis (data not shown).

Variation of observed peptides within serum

Previous studies have demonstrated that thermal denaturation of α thrombin occurs rapidly and follows first-order kinetics at temperatures above 54°C (18–20). Therefore, prior to time-course studies, serum samples from five subjects were divided in two sets, one of which was heated to 65°C for 30 min (maximum temperature and time allowing thermal inactivation without causing denaturation-mediated precipitation in the samples) in an attempt to reduce the intrinsic proteolytic activity. Following this, each serum set was subjected to incubation at room temperature for various times up to 2 h, and processed and analyzed by MALDI MS as described above. The data, including all time points and all subjects, are averaged into a single intensity for each unique FPA peptide, with each point and associated error bar intended to represent the variability of that particular peptide across time and between subjects (Figure 1). The 1465 m/z peptide was used to normalize the MALDI spectra prior to acquiring the relative peptide intensity and averaging the data. Examination of the neat serum samples reveals a time-dependant variation in the observed peptidome, especially evident with the high abundant FPA peptides. By comparison, the heat-inactivated samples display a reduction in variability in FPA content, consistent with the assertion that heat would denature most proteolytic enzymes and thus significantly reduce time-dependent variability. Further, MALDI has an inherent signal variability of as much as 20%, which may contribute

significantly to the observed FPA variability in heated samples.

Broad proteolytic damage caused by thrombin activity

To observe a more direct effect of the potential proteolytic activity of α thrombin on blood proteins, specifically isolated from the effects of the entire coagulation cascade, LMW proteins were isolated from plasma and subjected to enzymatic proteolysis. Either α thrombin or trypsin was added, the latter serving as a positive control for intentionally introduced proteolysis (standard "bottom-up" proteomics protocols). Isolation of proteins between 3 and 30 kDa, prior to digestion, is important for two aspects of this experiment: 1) fibrin is removed, so that the plasma will not "clot" upon addition of thrombin, and 2) since we monitor the evolution of peptides as the products of proteolysis, it is important to purge below 3 kDa, so that any peptides that are detected in the final analysis should result primarily from the intentionally induced enzymatic cleavage. The resulting digestions were subjected to RP-HPLC for examination (Figure 2).

In Figure 2A, the peptides/proteins present in the LMW are visualized at time 0, with the expectedly minimal peptide content from the sample preparation process described above. All of the chromatograms display peptide separations from 10 to 60 min elution, with this view omitting the large peak eluting at approximately 70 min containing the bulk of the intact proteins and large polypeptides. The resulting proteolysis after 7 days incubation (Figure 2B) is visualized by an increase in the number and intensity of peaks seen on the HPLC chromatograph. As expected, the

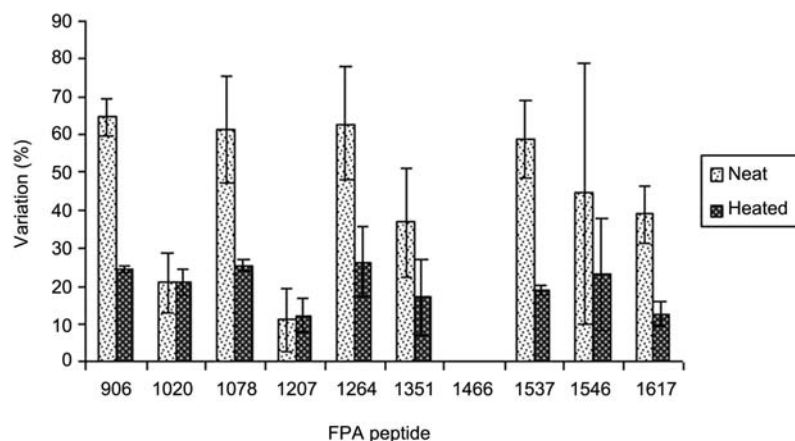


Figure 1 The variability of FPA peptides observed within serum samples across a 2-h time course from five subjects is graphically represented.

Light bars represent neat serum incubated at room temperature and sampled at 0, 15, 30, 60, and 120 min. Dark bars represent heat-treated aliquots of those same neat serum samples (mentioned above) sampled at the same time points. Time 0 is defined as immediately following clotting and initial centrifugation processes. The bar heights represent the average of intensity change over time 0, with all time points and all samples being averaged together into one value, with associated standard deviations as shown. Individual spectra were first normalized against the *des* Ala FPA peak m/z 1465.9. This simplified view allows a quick visual assessment of the variability seen over time, with a clear trend toward reduced variability in the heat-treated serum sample.

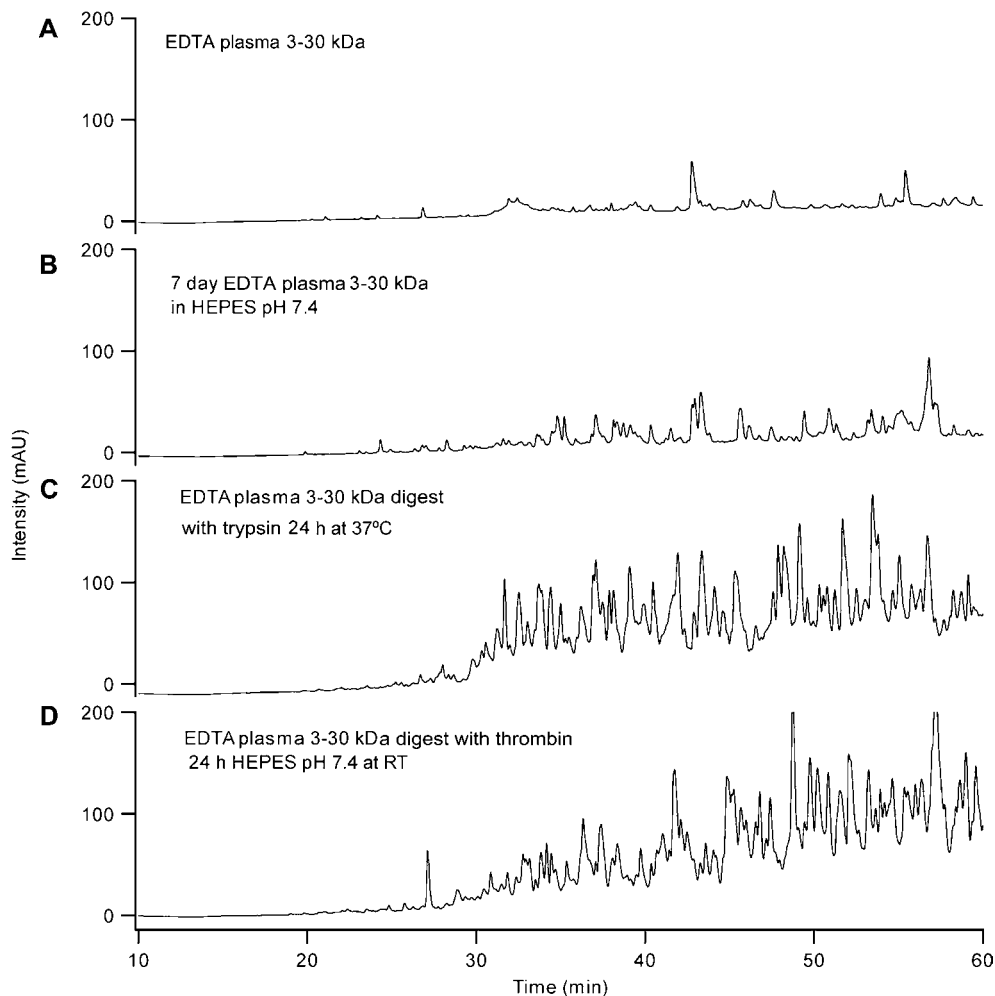


Figure 2 Examination of the in vitro proteolytic activity of α thrombin on plasma-derived proteins.

The low molecular weight (LMW) proteins (3–30 kDa) were isolated from plasma and subjected to in vitro proteolysis by α thrombin or trypsin. Peptide content, both before and after intentionally introduced proteolysis, is evaluated simply by the RP-HPLC chromatograms. (A) RP-HPLC separation of LMW proteome from plasma (EDTA) at time 0. Even after peptide depletion, some peptide peaks are evident in the starting material, but this view provides an initial baseline. (B) The prepared sample after dwelling for 7 days at room temperature (RT), without any added proteases. Some proteolysis is observed, with this view serving as a stringent negative control for the extrinsic digestion. (C) Trypsin digestion, for 24 h, of the LMW proteome. As would be expected, extensive proteolysis is evident. (D) α Thrombin digestion, for 24 h, of the LMW proteome. A peptide population of similar complexity to that produced by trypsin is evident.

added proteases (Figure 2C and 2D) lead to vastly increased peptide content. The results suggest that α thrombin can digest blood proteins with a similar efficiency to trypsin.

The effluent from these HPLC experiments was spotted onto a MALDI target at 15 s intervals for 60 min, and peptides were detected in MS mode, with parent ions selected based on their signal-to-noise (S/N) for TOF/TOF analysis. Peptides that were identified following α thrombin digestion all have R or K carboxyl termini as expected and sequence-based determinations of the parent protein were made (Table 2). The identified protein substrates are not functionally involved in coagulation, thus demonstrating enzymatic activity of thrombin clearly outside of its defined “specific” role within the clotting cascade. Significantly, only four out of the 32 cleavage sites represented by these peptides have the expected R-G

motif fitting the presumed “specificity” of α thrombin. Further, included in these peptides are three instances of missed R-G potential cleavage sites.

Thrombin digestion of hemopexin

Hemopexin (UniProtKB/Swiss-Prot-P02790) was selected from the non-coagulation proteins identified in Table 2 as a test case for validating the results of that experiment. Dilutions of partially purified hemopexin (from human plasma, >50% pure) were incubated with 0.1, 1, or 10 U of α thrombin per mL for 16 h and the peptides were examined by MALDI MS. In general, the same hemopexin peptides were generated at all three dilutions with all concentrations of α thrombin, though not uniformly detected throughout all replicates (data not shown). This single-substrate experiment facilitates detection of more

Table 2 Peptides evolved from α thrombin digestion of plasma proteins.

Protein (GenBank ID)	Peptide(s)	Score
Complement comp. C4A (gi 179674)	K_VGGNSKGLTKVLR_T	388
	K_SHALQLNNRQIR_G	
	R_NGFKSHALQLNNR_Q	
	R_NGFKSHALQLNNRQIR_G	
	R_QIRGLEEEELQFSLGSKINVK_V	
Lipoprotein CIII (gi 224917)	R_TLEIPGNSDPNMIPDGFNSYVR_V	301
	K_TAKDALSSVQESQVAQQAR_G	
Kininogen-1 precursor (gi 125507)	K_HATKTAKDALSSVQESQVAQQAR_G	192
	K_RPPGFSPFR_S	
Hemopexin (gi 386789)	R_GHGLGHGHEQQHGLGHGHKFKI ^a	111
	K_NFPSPVDAAFR_Q	
	R_FDPVRGEVPPR_Y	
PK-120 precursor (gi 1402590)	R_FDPVRGEVPPRYPR_D	101
	R_QLGLPGPPDVPDHAAYHPFR_R	
Unnamed protein (gi 36575)	R_TSAGTRQPQFISR_D	82
Apolipoprotein E3 (gi 1942471)	K_VEQAVETEPEPELR_Q ^b	70
KIAA1319 (gi 7243019)	R_ERELRALKGALK_E	68

LC-MALDI MS, following the RP-HPLC traces shown in Figure 2, yields identification of the peptides listed above, and enough sequence information to give highly confident identification of the parent proteins. All peptides display the expected R or K carboxyl terminal residues (marked by underscore). Mascot scores are listed for the parent protein identification. ^aC-terminal fragment. ^bN-terminal fragment.

Table 3 Digestion of hemopexin by α thrombin.

Peptide mass	Position	Missed cuts	Peptide sequence
1129.65	379–386	1	R_RLWVLDLK_S
1220.60*	92–102	0	K_NFPSPVDAAFR_Q ^a
1268.67*	209–219	1	R_FDPVRGEVPPR_Y ^a
1402.77	116–126	2	K_VWVYPPEKKEK_G
1442.77	103–115	1	R_QGHNSVFLIKGDK_V
1517.78	81–91	2	K_WDRELISERWK_N
1534.78*	90–102	1	R_WKNFSPVDAAFR_Q
1555.79	78–89	2	K_SHKWRELISR_W
1684.89*	209–222	2	R_FDPVRGEVPPRYPR_D ^a
1837.88	387–402	0	K_SGAQATWTELPWPHEK_V
1999.91	223–239	2	R_DVRDYFMPCPGRGHGHR_G ^b
2344.22	92–112	1	K_NFPSPVDAAFRQGHNSVFLIK_G
2441.29	103–123	2	R_QGHNSVFLIKGDKVWVYPPEK_K
2644.36	92–115	2	K_NFPSPVDAAFRQGHNSVFLIKGDK_V

Evolved peptides were identified by peptide mass fingerprinting. Peptides able to be confirmed by high-quality TOF/TOF spectra are indicated by (*) after the determined mass. A count of missed or incompletely cut R/K potential cleavage sites is provided. Note that only one cleavage site (marked with 1) out of the 28 detected cleavages has the “specific” R-G motif typically associated with thrombin cleavage of fibrinogen. By contrast, there are three instances (underlined) that bear the R-G motif but were not cleaved by thrombin. ^aThe three peptides identified from plasma protein digestion in Table 2. ^bPutative thrombin recognition site R-G.

peptides generated by α thrombin digestion (Table 3), and includes all of the hemopexin-derived peptides detected from the plasma protein digestion (Table 2). Consistent with Table 2, all peptides share either R or K carboxyl termini, but seemingly lack any preference for the other residue of the scissile bond. We again note several missed R-G cleavage motifs. These targeted results further support our hypothesis that the substrate specificity of α thrombin is not limited to coagulation related proteins.

Discussion

FPA peptides in serum

While the dominant presence of FPA is expected in a serum sample, the abundance of truncated FPA frag-

ments in serum is a corollary feature (15). A direct comparison of equal amounts of EDTA plasma failed to reveal an equivalent FPA fragment profile, even after applying the more sensitive LC-MALDI analysis, suggesting generation of the truncation pattern is itself unique to serum. In fact, it has been demonstrated that in vitro clotting of purified fibrinogen (from human plasma) results in the release of four peptides, A(FPA), AP(FPA-P), Y(*des* Ala FPA), and B (FPB). FPB accounted for approximately one-half of the total mass. The remaining peptides consisted of the three FPA fragments: FPA –70%, FPA-P –20%, *des*Ala FPA –10% (21). Previous studies with fibrinogen have demonstrated that phosphorylation of the A α chain serine, a post-translational modification occurring prior to secretion from the liver (22–24), and removal of the amino-terminal alanine from the A α chain are

regulated with respect to subject parameters, such as age, trauma, or illness (24–27), and are in place on fibrinogen prior to thrombin action (22–24). The low levels of FPA present in EDTA plasma can be attributed to the expectedly minimal thrombin activity in vivo and minimal ex vivo thrombin activation accomplished by calcium sequestration by EDTA. In serum, however, thrombin derived FPA levels rise dramatically due to clotting, as expected, but also, somewhat surprisingly, NH₂ terminal truncated FPA peptides appear quite prevalently (Figure 1). This observation leads us to hypothesize that the obvious presence of amino-peptidase(s) activity may somehow be increased, either directly or indirectly, by activation of the clotting cascade, and may be adversely affecting the stability of other proteins and peptides. The amino-peptidase activity, seen here at the macro level, will degrade proteins and/or peptides at various rates, probably in a sequence-dependent manner, and both high and low abundance proteins/peptides may be susceptible to amino-peptidase degradation.

Variability of serum protein and peptide content

Serum is the liquid portion of a clotted blood sample. Clots form due to an enzymatic cascade dominated by proteases, including thrombin, but after clot formation is complete, these enzymes largely retain their proteolytic potential. Residual enzyme activity was demonstrated by showing that the multiple FPA fragments discussed above could be generated in vitro from extrinsic FPA (full-length) by the in vivo enzymes intrinsic to serum (6). In the current study, time-course experiments were conducted to see if the intrinsic proteolytic activity of serum can be monitored by time-dependant changes in the observed peptidome.

In fact, serum appears inherently variable as evidenced by MALDI MS spectra that were taken over specific time intervals (summarized in Figure 1). Unfortunately, previous reports have stated that the *des* Ala FPA (*m/z* 1465.9) is ionized far better than the full-length FPA (*m/z* 1536.9) (17) and thus is more easily detected in MALDI MS, such that the relative abundance of *des* Ala FPA cannot be judged by intensity in MALDI spectra.

We hypothesized that if the time-dependent variability of serum was indeed due to intrinsic enzymatic activity, that heating the samples, and thus denaturing the enzymes (18–20), should reduce the time-dependent variability in the samples. Previous studies (18–20), confirmed by our own initial data (not shown), indicate that 65°C for 30 min was effective for α thrombin denaturation. Therefore, aliquots of all samples were heated to 65°C for 30 min, and then examined across another time-course experiment for variations in the observed peptidome. Thermal treatment seems to reduce the variability of FPA markers in all subjects across the time-course observed, presumably reflecting reduced intrinsic protease activity (Figure 1).

A more direct examination of the general proteolytic activity of α thrombin was conducted by isolating

the LMW proteins (between 3 and 30 kDa) from plasma. Importantly, this size-discriminated protein fraction is depleted of fibrinogen, and therefore cannot clot upon α thrombin addition, thus facilitating specific examination of any proteolytic activity of α thrombin beyond its well-characterized and supposedly “highly specific” role in clot formation (2, 5, 6, 21, 28). Figure 2 clearly demonstrates that α thrombin activity results in significant plasma protein digestion, in fact on the same scale as trypsin; the latter being traditionally used for intentionally caused and extensive protein degradation typical of “bottom-up” proteomics experiments (29). Thrombin clearly exhibits extensive proteolysis upon the blood proteome, belying the biological “specificity” of this protease.

Biologically, thrombin cleaves fibrinogen, removing the N-terminal “caps” to unmask fibrin self-assembly potential (7). Cleavage of the A chain occurs between residues Arg16 and Gly17 thus releasing the 16 residue FPA peptide (6). The perceived narrow substrate range and clinically relevant “enzymatic specificity” of thrombin may possibly be attributed to the fact that it cleaves only four of the 376 trypsin-susceptible sites in fibrinogen (6, 28). With fibrinogen as a substrate, thrombin activity appears specific to only R-G sequences, but, unlike trypsin, thrombin has been shown to release the amino-terminal arginine, in an exopeptidase-like fashion, even if the arginine is NO₂ protected (28).

Table 2 displays the list of peptides identified by TOF/TOF sequencing, as well as the parent protein, following digestion of the peptide-depleted LMW plasma proteins with α thrombin (from Figure 2). Essentially, these results demonstrate a bottom-up proteomics experiment enabled by α thrombin instead of trypsin. All peptides show the R or K carboxyl termini expected from α thrombin digestion. Two features from this Table are notable. First, the parent proteins listed are not functionally exclusive to coagulation or fibrinolysis. This observation is consistent with previous studies, where α thrombin is implicated as a proteolytic agent in other biological processes (30–32). Second, only a very few of the cleavage sites match the R-G motif commonly expected for α thrombin, and there are a few missed R-G sites included in the observed peptides. These data demonstrate in vivo catalytic potential of α thrombin as a surprisingly non-specific trypsin-like serine protease, lacking both the substrate specificity (toward fibrinogen) and the sequence specificity (R-G sites) often associated with the well-known clotting function of α thrombin.

To further validate that α thrombin activity was responsible for this observed degradation, plasma purified (>50%) hemopexin was selected as a single specific substrate. Peptide and protease-free hemopexin was prepared, and incubated with increasing amounts of α thrombin. The resulting peptides (Table 3) confirm that hemopexin is cleaved by α thrombin. Importantly, this list includes the peptides already detected from α thrombin digestion of the more complex LMW plasma protein sample (Table 2), indicat-

ing, by extension, that the other proteins were also substrates for α thrombin. As before, the majority of cleavage sites do not have the R-G motif, while several R-G sites within the peptides were not cleaved.

Conclusions

Unlike previous reports claiming the R-G cleavage specificity of α thrombin (for fibrinogen) (21, 28), we observed a much broader tolerance for residues on the carboxyl side of the scissile bond. However, the data suggesting a limitation to R-G cleavage specificity must take into account the given experimental conditions (21, 28). Some of the peptides we observed derive from parent proteins with molecular masses much higher than our "sieving" process (3–30 kDa) should have allowed. This can be explained both by mild imprecision of the filtration device, and, more importantly, by proteolysis having occurred within the starting EDTA plasma itself, resulting in large fragments within the selected mass range (33).

Thrombin may have a broad spectrum role in cellular responses. The presence of thrombin receptors seems ubiquitous on cell membranes, and thrombin itself (when binding to such) appears to mediate a multitude of responses from cellular proliferation to cytokine release (34). It is suggested that thrombin activity is due to local synthesis of activators of the "prothrombinase complex", such as tissue factor VIIa and γ -glutamyl carboxylase, and possibly has a role in folliculogenesis (34). Taken together, it would seem that, in a biologically uncontrolled environment, such as inside a blood collection tube, α thrombin can induce a variety of biological processes, perhaps especially in the presence of cells in whole blood or residual cells in serum or plasma. The subsequent proteolytic activity observed with substrates beyond fibrinogen thus may become kinetically favorable especially over prolonged periods in the tube. By extension, it has also been suggested that the degree of proteolysis is indicative of general health and the peptides generated may have value in biomarker discovery (14, 15, 35).

Our data suggest that the presence and activity of α thrombin, directly or indirectly, decreases protein stability, and thus reduces the utility of serum as a source for protein biomarker discovery. Enzymes in the clotting cascade, though specific in biological role, have been shown to have promiscuous activity (4, 5, 30–32), outside their expected catalytic function. The data presented here provide direct evidence of this substrate promiscuity, acting broadly upon the protein and peptide content of the plasma/serum itself.

We provide evidence that serum sample variability is influenced by intrinsic proteolysis (specifically α thrombin) and can be reduced by heat inactivation. In general, broader protease inhibition (using chemical inhibitors) or inactivation by heat treatment may be warranted in cases where sample stability is desired.

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