

Controlling the Major Factors Impacting Trypsin Digestion

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Section 1: Introduction

It is rarely the case that an analyst is presented with a sample in a suitable form for injection into an LC/MS system. Often the sample must be manipulated to concentrate the compound of interest, remove interferences, enzymatically digest the sample to generate surrogate peptides with better mass spectrometric properties, and modify the sample matrix or buffering system to achieve compatibility with LC separation and mass spectrometric detection systems.

Method development is time consuming and digestion is one of the largest sources of variation in sample preparation. The drop in substrate concentration as the reaction progresses leads to asymptotic product formation. Furthermore, trypsin autolysis results in a loss of specificity and sample contamination precluding the use of high concentrations of enzyme (1). While the reaction kinetics are a limiting factor, the digestion process is further mediated by protein denaturation, peptide degradation, and enzyme activity. These factors can also be impacted by solubility and diffusion. In turn, implementation is governed by cost as well as ease of use. Balancing these factors is essential.

Section 2: Activity

- I) Optimization using of sequencing grade soluble trypsin**
- II) Optimization using of soluble porcine trypsin**
- III) Optimization using an immobilized enzyme reactor**

Optimization using of sequencing grade soluble trypsin

Trypsin digestion follows Michaelis-Menton kinetics. Theoretically, while substrate concentrations remain stable the reaction rate is linear. As the reaction proceeds there is a drop in substrate concentration and therefore a decrease in the reaction rate. Also, as the reaction proceeds trypsin autolyzes and there is a loss of specificity (1) such that tryptic peptides are cleaved with non-tryptic specificity, leading to a loss in signal that increases with further digestion. This can be seen in the attached Supplementary Data.

Using insulin (5.8 kD molecular weight) as a substrate and sequencing grade modified trypsin as a model system, you see the characteristically linear product formation during the early stages of the reaction followed by asymptotic product formation. This reaction is readily accelerated by increasing the enzyme concentration. Using a 50:1 protein:enzyme ratio you have a complete digestion after 18 hours. Using a 5:1 protein:enzyme ratio you have a complete digestion of insulin in 3 hours (see Supplementary

Data - Activity). However, insulin is one of the easiest to digest proteins, containing only two cleavage sites, both of which are readily available to trypsin. Attempts to apply this strategy to highly structured proteins often results in incomplete digestion. For example, a more complete digestion of C-reactive protein results in a 10 fold increase in signal over a 48 hour digestion with 2 additions of trypsin at a 60:1 ratio (see Supplementary Data - Activity). Furthermore, while digesting a 400uL sample of 100ug/mL insulin can be cost effective, application of modified trypsin to a complex sample can become costly. Digesting a 5uL sample of plasma using a 5:1 protein:enzyme ratio can cost more than \$30 per sample.

Optimization using of soluble porcine trypsin

To lower the costs associated with these reactions, animal-derived pancreatic trypsin is being used with increasing regularity. As a result, there is little change in digestion time but effectively a 1000x reduction in enzyme cost. If you compare the use of 2 sources of trypsin at 50:1 protein:enzyme ratios the results look almost identical. However, as you increase the concentration of enzyme you begin to see significant product degradation caused by unmodified trypsin. This phenomenon has been fairly well characterized (1). Trypsin in solution is prone to autolysis and corresponding loss of specificity.

Optimization using an immobilized enzyme reactor

Immobilization restricts trypsin-trypsin interactions and therefore inhibits autolysis. Immobilization thus enables the use of very high concentrations of enzyme with enzyme activity and specificity being conserved throughout the duration of the reaction. Additionally, immobilization, like some chemical modifications, adds a limited amount of thermal stability, generally increasing the optimal temperature for digestion by 5-10°C.

Section 3: Denaturation

- I) Impact of Common Denaturants**
- II) Use of Thermophilic Trypsin to Accelerate Digestion/Denaturation**

Optimization of enzyme activity is essential to overcoming limitations associated with enzymatic digestion. However, the benefits of increased activity are insignificant unless cleavage sites are accessible. Some proteins are relatively planar, while others are highly structured. Others have localized domains of varying structure. As structure increases, denaturation plays an increasingly prevalent role in complete digestion.

While trypsin digestion protocols have remained relatively static over time (only changing enzyme to protein ratios, reaction times, and trypsin source – modified, recombinant, or animal derived), denaturation protocols vary significantly including not only concentrations, duration, and denaturant source, but also type of denaturant, temperatures employed, and co-denaturants added.

Chaotropes, surfactants, detergents, organic solvents, pressure, microwaves, ultrasound and temperature techniques have been applied with varying success to increase denaturation and rate of digestion and digestions are often performed in the presence of chaotropes, surfactants, salts and organic solvents. Due to the general robustness of trypsin it is often assumed that activity is negligibly affected by these additives. However, we have determined that trypsin activity varies significantly depending on the buffer used (see Supplementary Data - Denaturation).

As with any protein, trypsin structure and function are interdependent such that most methods used to disrupt protein structure as part of the sample preparation process coincide with a loss of trypsin activity. Stabilizing trypsin to make it resistant to denaturation at elevated temperatures, then operating above the melting point of the proteins of interest provides a simple yet effective means of denaturing proteins while maintaining robust trypsin activity.

Thermophilic enzymes like Taq (*Thermus aquaticus*) polymerase have amino acid sequences that enable improved structural stability. In general this means a hydrophilic exterior and hydrophobic interior. Studies indicate the mesophilic characteristics of trypsin result from a non-optimal structure [2]. Methods developed at Perfinity dramatically improve the structural stability of trypsin. Large hydrophilic polymers, sized so as to preclude access to the interior of trypsin are used during the immobilization process. Then internal residues treated to be made more hydrophobic. The resulting enzyme exhibits no change in specificity and a significant increase in thermal stability.

In addition to the kinetic acceleration predicted by the Arrhenius equation, digestion at elevated temperatures enables simultaneous denaturation and digestion. This results in significant time savings, increase in peptide signal and simplification of the workflow. When comparing results obtained at 40°C and 70°C, a 1000 fold increase in peptide formation is readily observed (see Supplementary Data - Denaturation).

Section 4: Solubility

Sample pretreatment at elevated temperatures to promote denaturation is the norm. Standard practice calls for denaturation and reduction performed at temperatures at 60°C for 1 hour. Increased temperature also increases solubility of hydrophilic proteins and peptides; however it has the opposite effect on hydrophobic specimens. This is usually mitigated by the use of high concentrations of chaotropes and surfactants, but given the negative impact of chaotropes and surfactants on activity it is desirable to find an alternative reagent enabling operation at elevated temperatures while promoting solubility even when handling high concentrations of protein or hydrophobic specimens. This led us to the use of glycerol in our digestion buffer instead of the application of a chaotrope or surfactant that might have significant deleterious effects on our digestion. A large number of additives were screened to determine their impact on solubility (see Supplementary Data – Solubility).

Section 5: Diffusion

Immobilization of an enzyme on a support can have many different impacts upon the reaction rate. One key factor is the potential introduction of diffusion limitations that potentially limit reaction rates. Shaking and utilization of stationary phases with large pores dramatically mitigate diffusion limitations associated with the use of immobilized enzymes.

Section 6: Incorporation of Affinity Selection into the Accelerated Workflow

Low abundance proteins require enrichment prior to analysis. As such it was important to provide a simple means of incorporating affinity selection into the workflow. This was achieved by means of a co-immobilizing streptavidin and a heat activated version of the temperature stable immobilized enzyme. During affinity selection the enzyme is kept inactive. Following purification the enzyme is activated by means of increased temperature and remains active under protein denaturing conditions.

We intend to expand further on this subject in a follow-up white paper. For additional information in the interim feel free to email us at info@perfinity.com

Section 7: Simplification of Workflow using Improved Magnetic Beads

In order to simplify sample handling magnetic beads were developed specifically for use as part of mass spectrometric sample preparation. Mass spectrometry is non-amplified (unlike PCR or LBAs) and you can't read in place; you have to inject the sample. As a result, workflows require stationary phases with high surface area/activity and high recovery. These features are often orthogonal. However, the same coatings that enable the development of the temperature stable immobilized enzyme reactor proved invaluable in the development of magnetic beads materials with these desired characteristics.

We intend to expand further on this subject in a follow-up white paper. For additional information in the interim feel free to email us at info@perfinity.com

Summary

In order to achieve greater results with trypsin digestion, it is necessary to have a full understanding of how the various factors impacting the digestion interact. Immobilization provides an intuitively simple means of inhibiting trypsin-trypsin interactions and providing an extremely active reaction system. Thermally stable trypsin enables the use of various temperatures to increase the speed and quality of results, while offering the ability to remove the need of agents that can harm the digestion process.

Supplementary Data

Supplemental Data - Section 2: Activity

Supplemental Data - Section 3: Denaturation

Supplemental Data - Section 4: Solubility

Supplemental Data - Section 5: Diffusion

Supplemental Data - Section 2: Activity

In order to determine the impact of enzyme:substrate ratio on trypsin digestion, an assay was developed using a digestion of insulin. Insulin provides a better indication of trypsin activity than small molecule surrogates such as BAPNA, as it relates directly to protein digestion. Furthermore, insulin creates a very simple peptide map and has several chymotryptic sites that will produce fragments if the enzyme has any chymotryptic activity. Following incubation the digestion reaction was quenched by means of acidification. When exposed to trypsin, human insulin forms two major products. There is a C-terminal peptide sequence (R)GFFYTPK and a larger N-terminal sequence (see Figure 1). These two peaks are easily resolved from the intact protein using reversed phase chromatography (Figure 2).

Figure 1: The amino acid sequence of insulin



Figure 2: LC/UV analysis of insulin peptide map (partial digest)

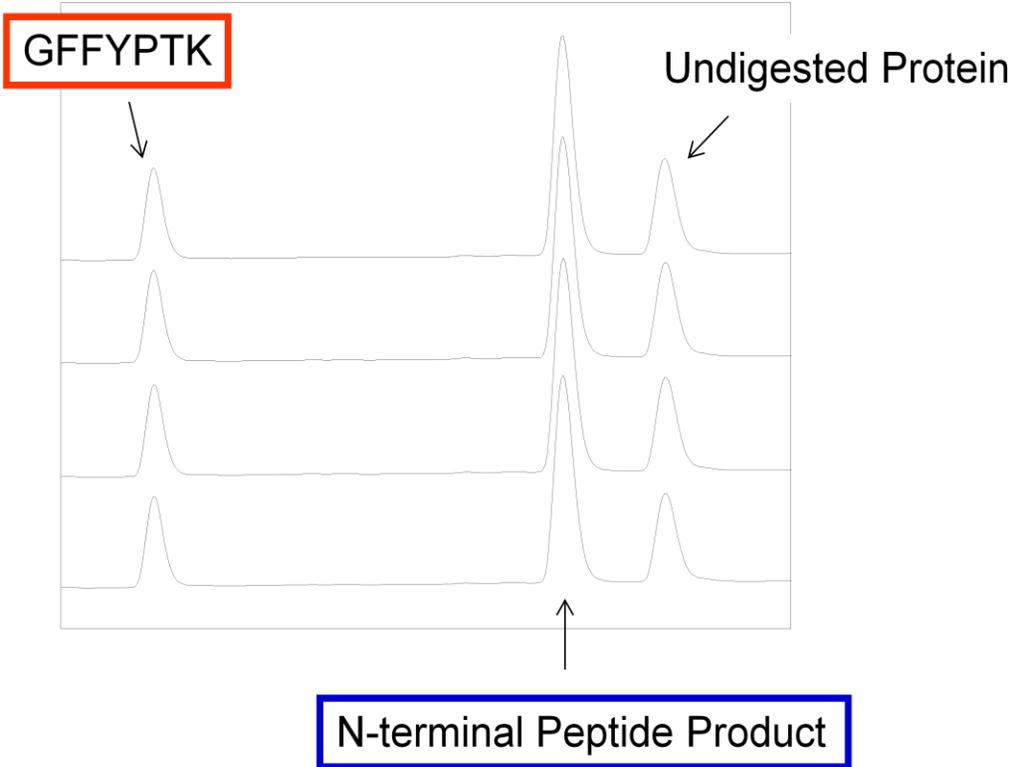


Table 1: A Summary of Insulin Digestion Times as a Function of Trypsin Concentration

Digestion of 400uL, 100ug/mL Insulin	
Enzyme: Substrate Ratio	Digestion Time (hours)
1:5	3
1:10	6
1:25	16
1:50	18

Figure 3: Timecourse of Insulin Digestion using Soluble Sequencing Grade Trypsin

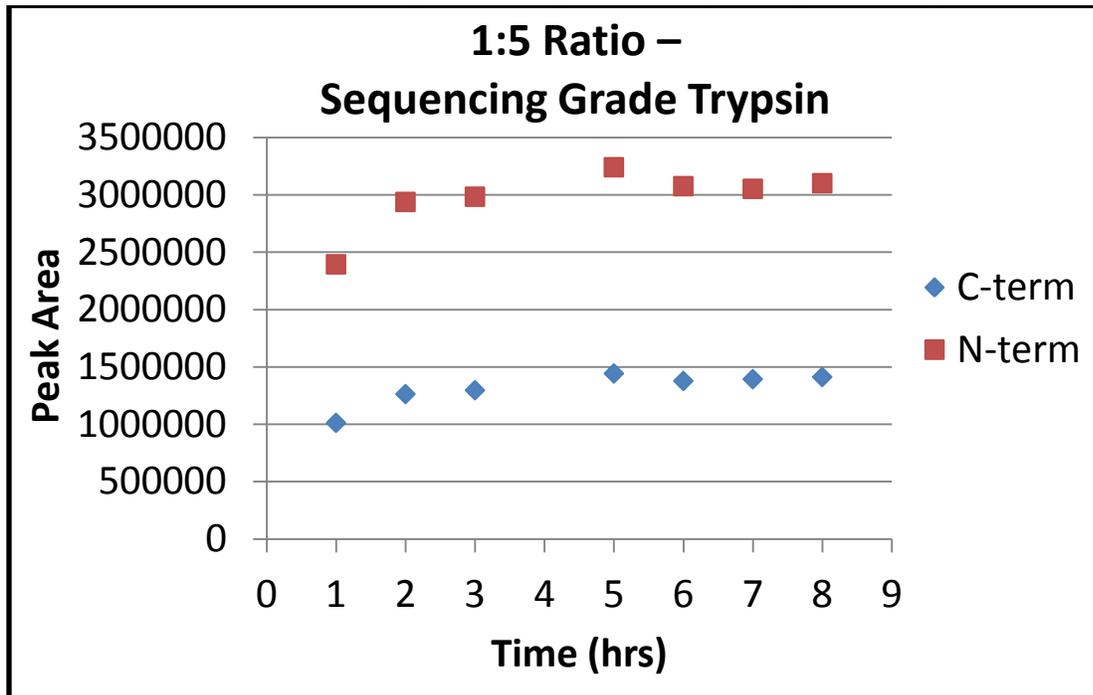


Figure 4: Timecourse of Insulin Digestion using Soluble Porcine Trypsin

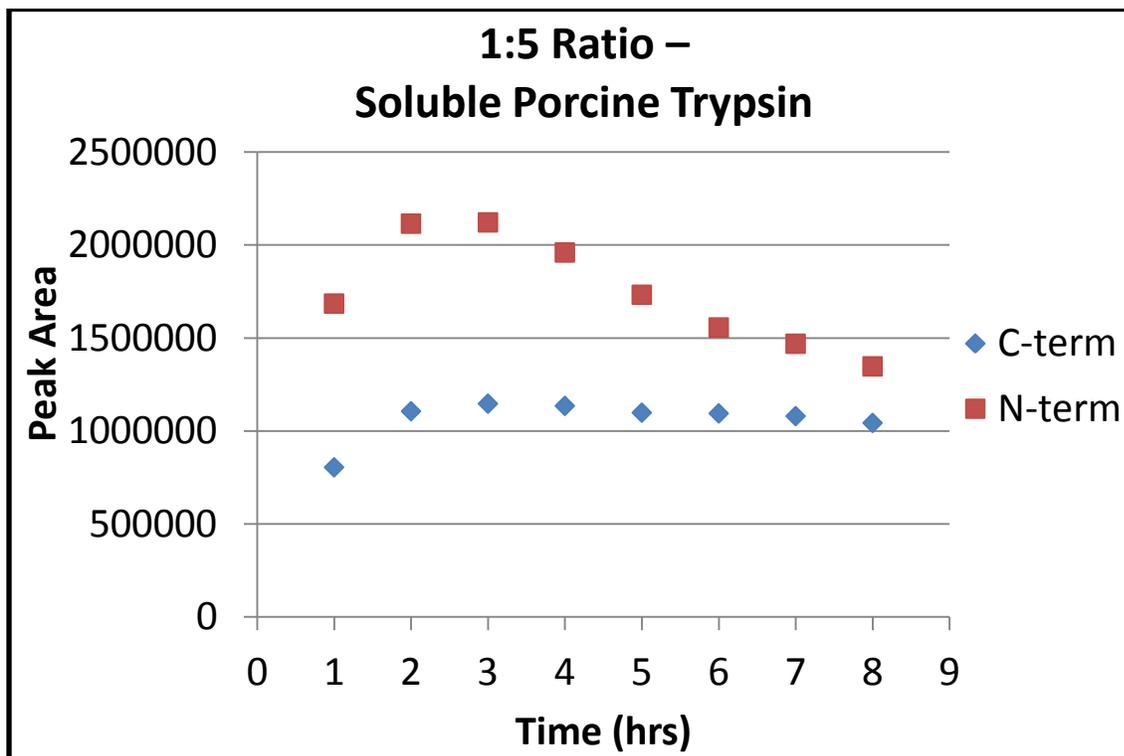


Figure 5: Peptide Maps of Insulin Digested using Soluble Porcine Trypsin

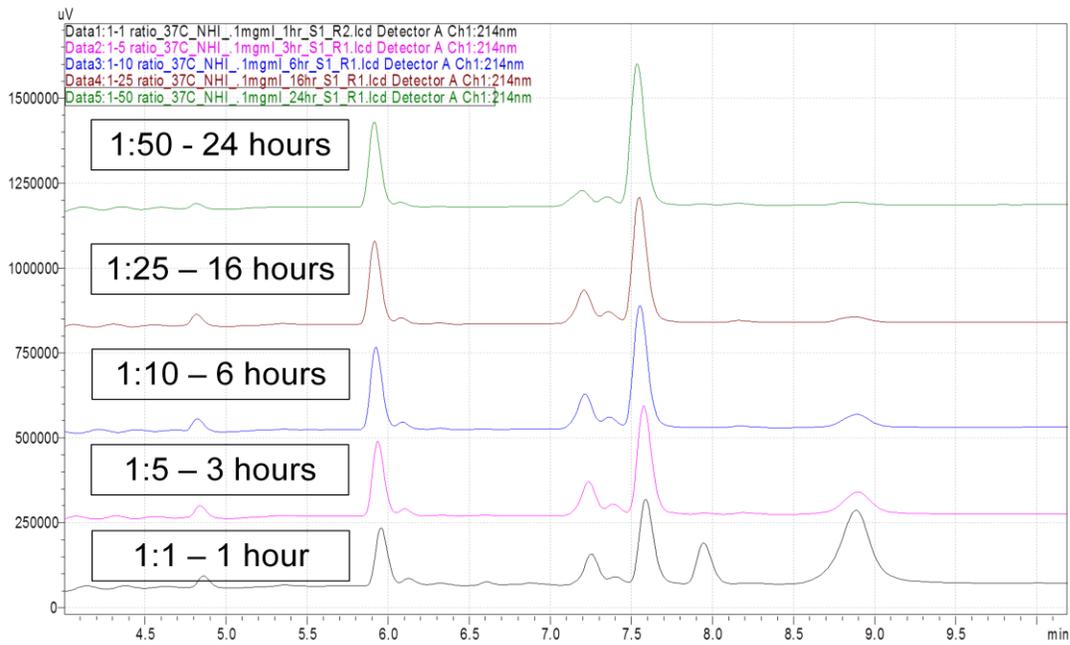
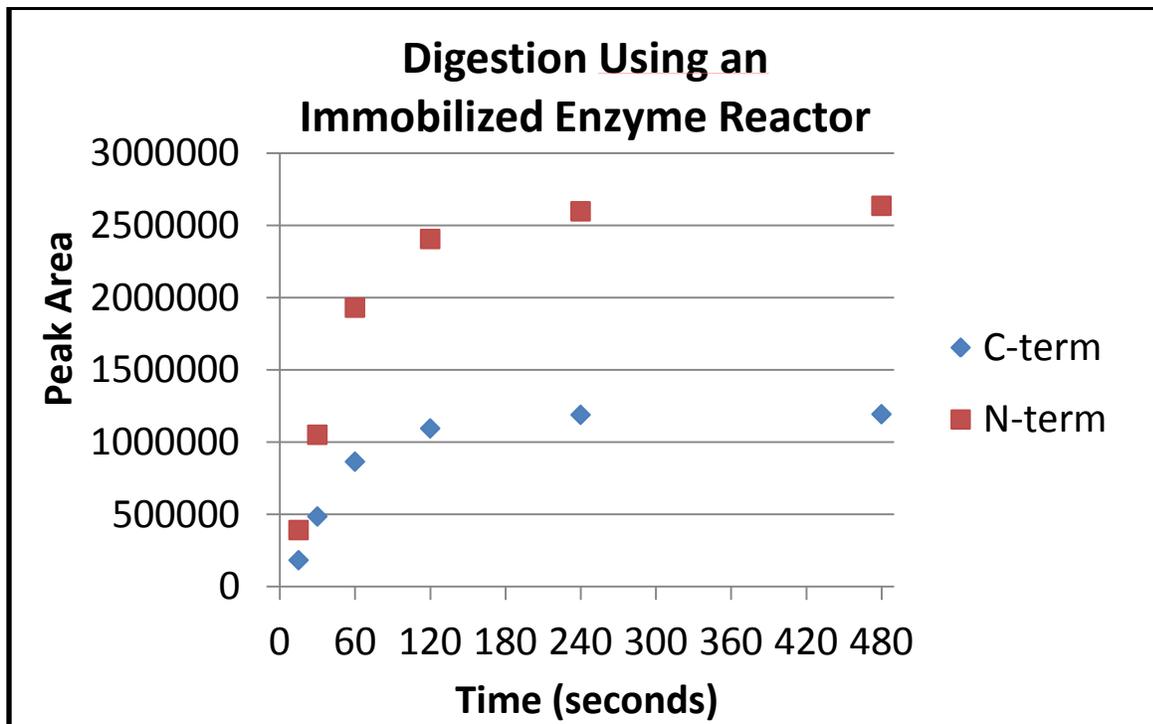


Figure 6: Timecourse of Insulin Digested using an Immobilized Enzyme Reactor



Impact of increased activity on sensitivity

Materials

Human C-reactive protein was obtained from Sigma (St Louis, MO). The Flash Digest kit is sold by Perfinity Biosciences (West Lafayette, IN).

Methods

SRM Development- Samples for SRM method development were prepared by adding 20µg of C-Reactive Protein (CRP) to a single well of the Flash Digest kit containing 150µL Flash Digest Buffer, 50µL water, and 0.1 wt% octyl-β glucoside. They were incubated at 70°C and 1400rpm on an Eppendorf ThermoMixer C (Hauppauge, NY) for 2 hours before sampling.

Digestion Optimization- Samples for the digestion optimization were prepared by adding 0.02mg of CRP to each well of a strip of Flash Digest containing 150µL Flash Digest Buffer, 50µL water, and 0.1 wt% octyl-β glucoside. They were incubated at 70°C and 1400rpm on an Eppendorf ThermoMixer C. Wells were sampled every 30 minutes, centrifuged and decanted prior to analysis by LCMS.

Comparison to Existing Methods³- 0.02mg of CRP was reconstituted in 100µL of 50mM Tris containing 5mM DTT and 2 g/L Rapigest. Samples were heated to 99.5°C for 5 min and then maintained at 60°C for 30 min. Samples were then cooled to room temperature (RT) and vortexed before the addition of 2µL of 750mM iodoacetamide. Samples were then incubated in the dark at RT for 1h. The alkylation was quenched by the addition of 2µL of 765mM DTT. 96µL of 50mM Tris with 5mM DTT was added to bring the sample volume to 200µL. At this point 0.33µL of freshly reconstituted trypsin (1µg/µL) in provided buffer was added (60:1 protein to trypsin ratio). The samples were incubated for 48h at 37°C with shaking. A second application of trypsin was employed at 24h. The reaction was stopped with the addition of 1µL TFA and then incubated for an additional 1h at 37°C before centrifugation and analysis of the supernatant.

Analysis

Samples were injected onto a Dionex Ultimate 3000 system equipped with a Halo C18 RP column (2.1x100mm, 2.7µm). Elution was performed using a gradient which consisted of Mobile Phase A – 98% water, 2% acetonitrile, 0.1% formic acid – and Mobile Phase B – 10% water, 90% acetonitrile, 0.1% formic acid. The gradient went from 2 to 50% Mobile Phase B over 60 minutes for the initial screen and covered the same range in 5 minutes for the later analysis. Peptides were eluted into a Thermo Velos Ion trap and were detected first using a Top Ten method and later an SRM based method.

Results

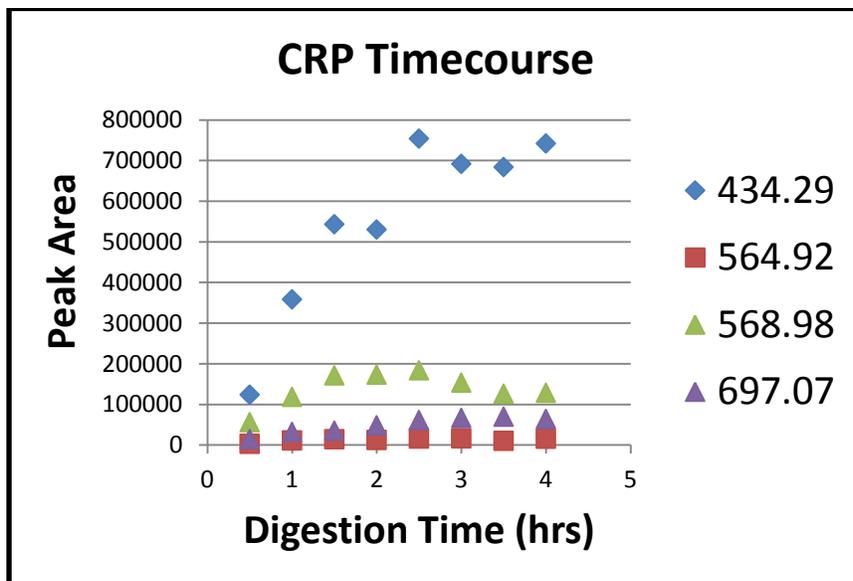
Based on our initial screening and a survey of the literature we focused on three peptides. The SRM transitions for each are listed in Table 2.

Table 2 Monitored peptide sequences for C-Reactive Protein

Peptide Sequence	Precursor Ion (<i>m/z</i>)	Fragment Ion (<i>m/z</i>)
APLTKPLK	434.29	398.76
ESDTSYVSLK	564.92	797.36
GYSIFSATK	568.98	716.34
QDNEILIFWSK	697.07	567.33

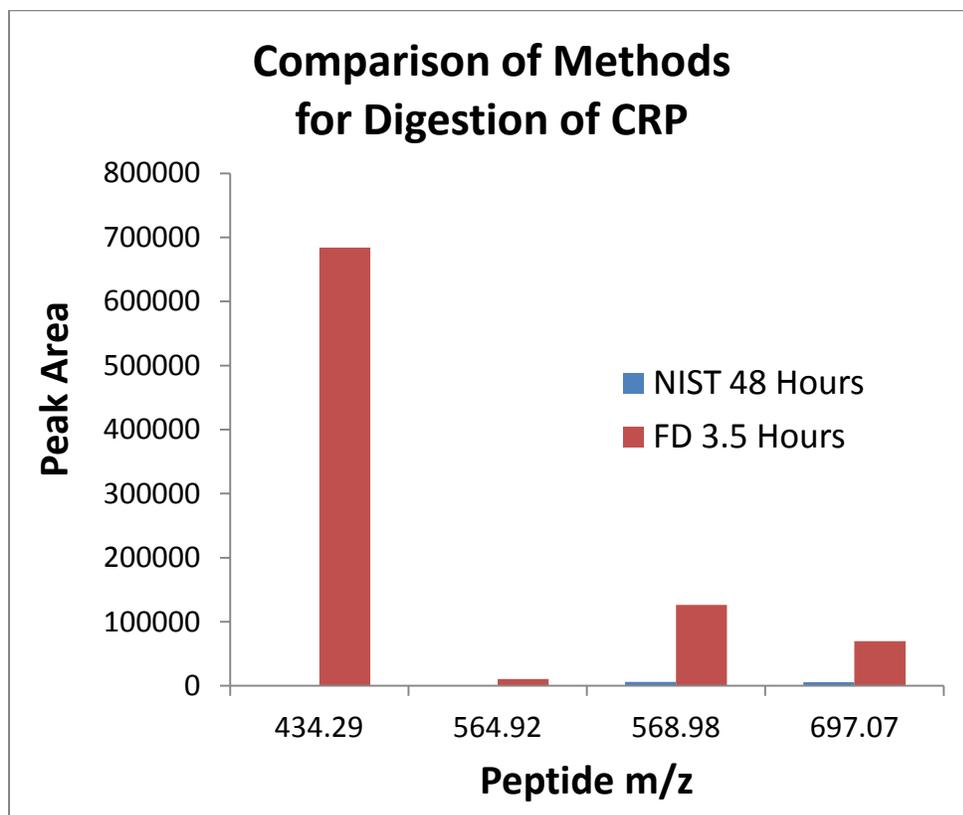
Running a digestion time course, we saw a maximum sensitivity obtained following 240 minutes of digestion using the temperature stable immobilized enzyme.

Figure 7 Digestion time course of CRP



Comparison work to other methods has shown less than one tenth the signal response for each of the four peptides observed. Based on previous experience with other digestion protocols and the relative extreme length of digestion time required to fully digest CRP we suspect that the lower signal achieved by these other methods is largely due to incomplete digestion of the protein.

Figure 8 A comparison of the Flash Digest protocol with a protocol found in the recent literature



Supplemental Data - Section 3: Denaturation

Digestions are often performed in the presence of chaotropes, surfactants, salts and organic solvents. Due to the general robustness of trypsin it is often assumed that activity is negligibly affected by these additives. However, it was determined in this study that trypsin activity varies significantly depending on the buffer used.

In order to determine the impact of various buffers and additives on trypsin digestion, an assay was developed using a partial digestion of insulin. The partial digestion was designed into the protocol to improve the quantification of variances. Following incubation the digestion reaction was quenched by means of acidification.

Method

100µg/mL solutions of insulin were made in a variety of buffers and additives. Except for the instances where the buffer itself was changed (e.g. for the testing of ammonium bicarbonate, PBS and HEPES) additives were in a solution of TBS, 10mM CaCl₂. These insulin solutions were then digested at 70°C using Flash Digest and a 1 minute digestion time. This provided an incomplete digestion.

Analysis

Insulin digestion products were analyzed by LC/UV using a 2.1x100mm ES C18 Halo column, a 20-70%B gradient in 7 minutes with the column oven set to 40°C (Mobile phase A= 2% ACN 98% water, 0.1% TFA, Mobile phase B= 90% ACN, 10% water, 0.1%TFA).

Results

It was found that the various additives and their concentrations can have dramatic effects on the capabilities of trypsin. There were a few additives that showed little impact, such as octylglucoside, but many caused a decrease in the degree of digestion, with some such as guanidine HCl and urea having very negative, albeit concentration dependent effects. It is worth noting that while high pH buffers outperformed Flash Digest buffer, deviations in the Flash Digest buffer pH away from pH 7.4 were not made so as to avoid potential increases in digestion buffer induced post-translational modifications. The effects of a wide variety of common chaotropes, surfactants, salts and organic solvents are shown in Figure 9.

Figure 9: (Part 1 of 2) peptide peak areas generated from Flash Digestion as a function of digestion buffer

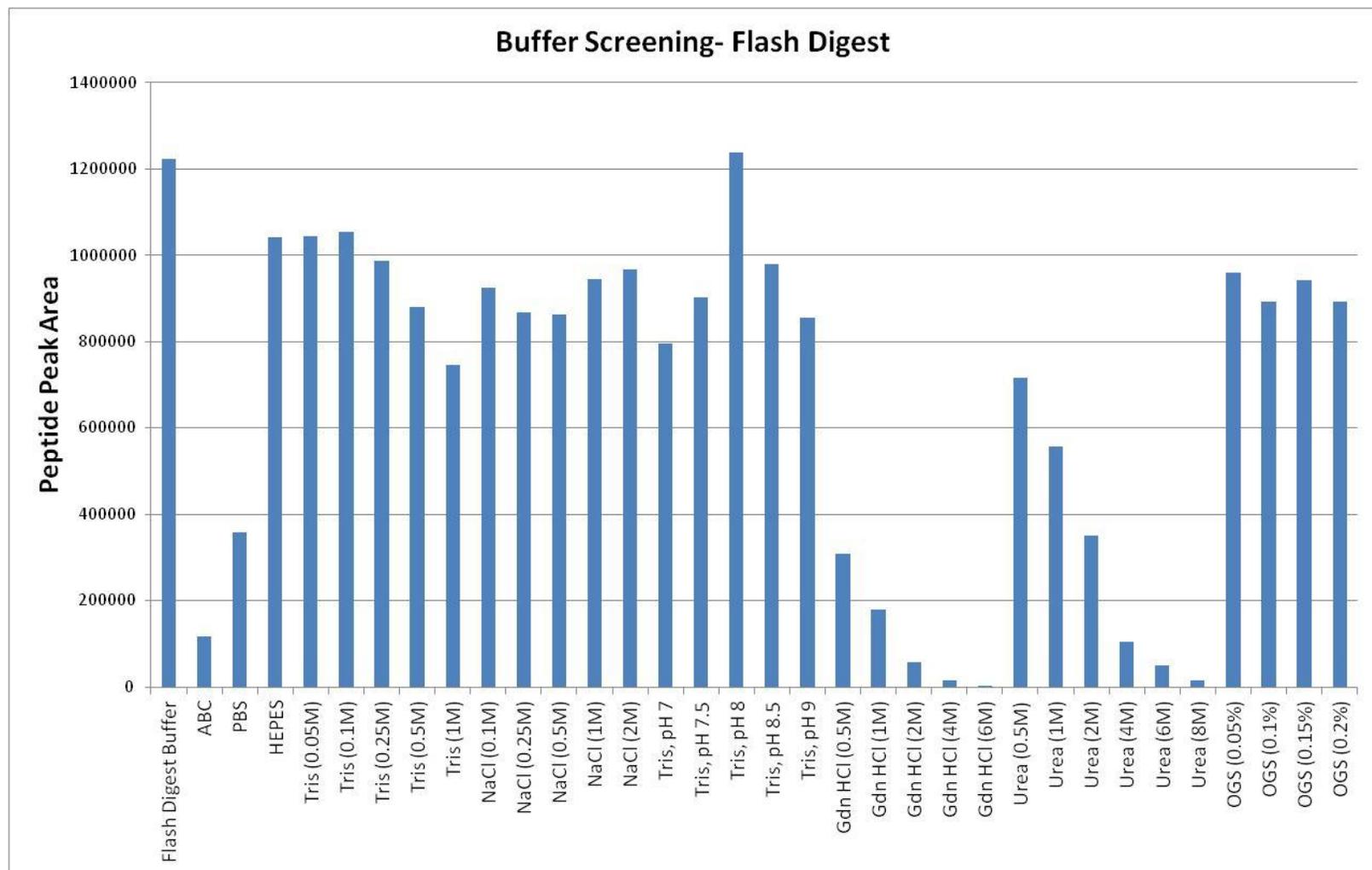
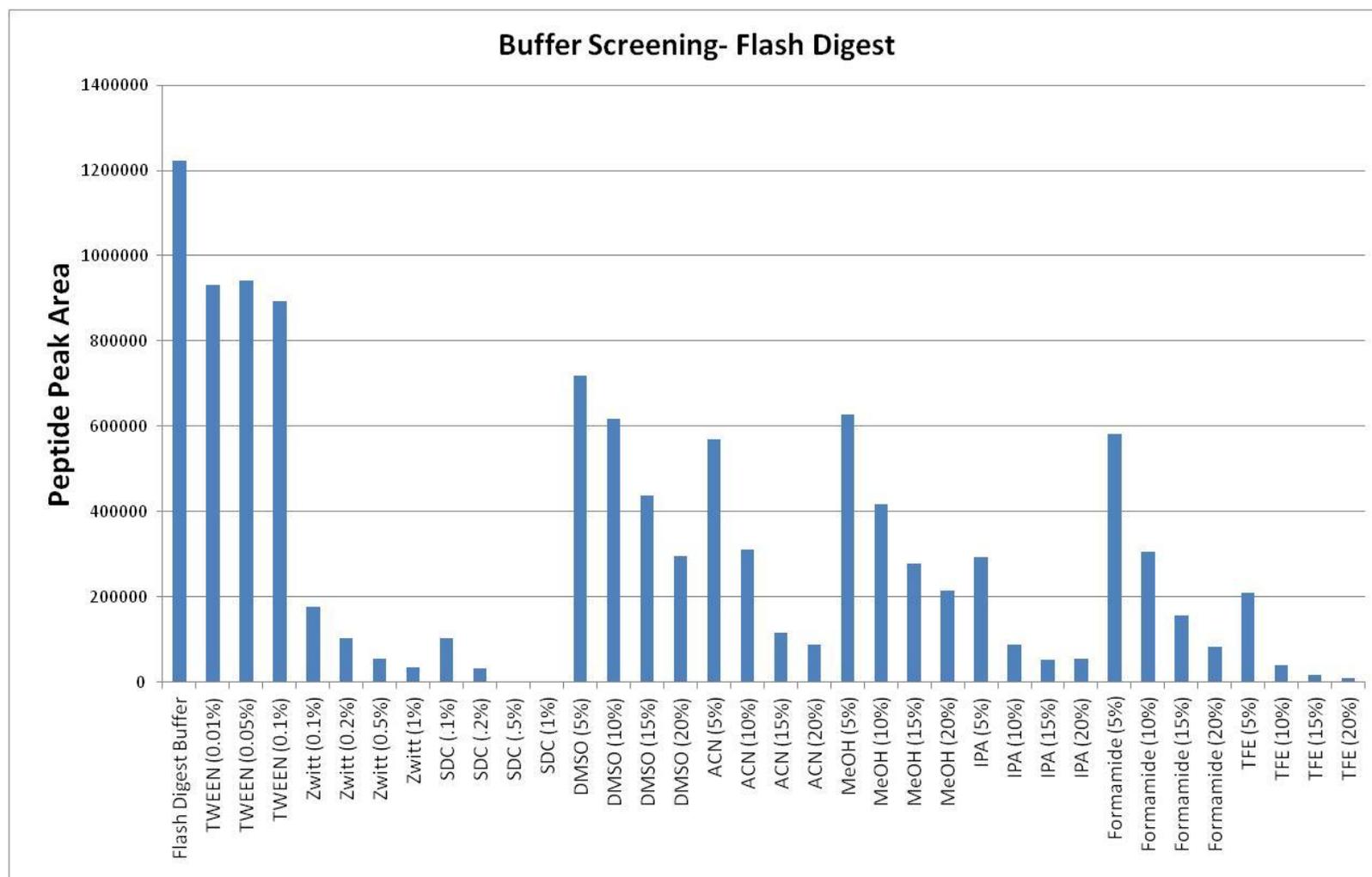


Figure 9: (Part 2 of 2) peptide peak areas generated from Flash Digestion as a function of digestion buffer



Thermophilic Trypsin Method

1µg/mL solutions of human IgG were made in Flash Digest Buffer. The temperature stable immobilize enzyme was synthesized and samples were added directly to this slurry without pretreatment (neither reduction nor alkylation were employed). Samples were heated using an Eppendorf ThermoMixer C equipped with a PCR block and a heated lid set to 70°C, 1400 RPM. Following incubation the entire sample was removed using a pipette and transferred to a clean Eppendorf tube, centrifuged, decanted and the supernatant analyzed by liquid chromatography/mass spectrometry.

Analysis

The digestion efficiency as a function of temperature was evaluated by measuring the peak areas corresponding to the two peptides VVSVLTVLHQDWLNGK and TTPPVLDSDGSFFLYSK. These two peptides are unique to human IgG. The peptides were analyzed by LCMS according to the parameters summarized in Table 3.

Table 3 The mass spectrometric parameters used for the analysis of the IgG1 digested at various times and temperatures using a temperature stable immobilized enzyme reactor

Sample	1ug/mL human IgG
Injection Volume	5 uL
Reversed Phase A	2% ACN (aq) 0.1% Formic Acid
Reversed Phase B	90% ACN (aq) 0.1% Formic Acid
Reversed Phase Gradient	2-70%B in 5 minutes
Sequence 1: MS1/MS2	VVSVLTVLHQDWLNGK- 603.82/805.62
Sequence 2: MS1/MS2	TTPPVLDSDGSFFLYSK – 938.02/805.26

Results

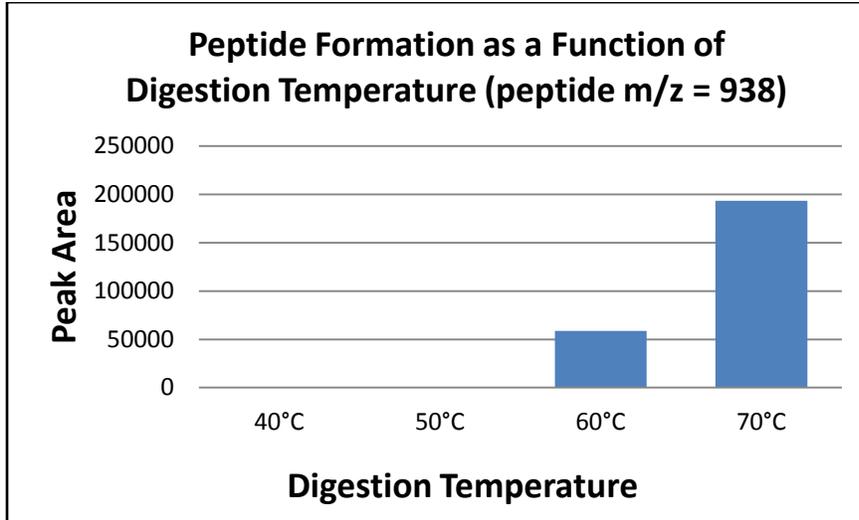
Table 4 Summarizes the peak areas associated with digestion of human IgG1 at various digestion times and temperatures while Figures 10 and 11 provide a visual representation of the increase in peptide generation as a function of increasing digestion temperature.

Table 4 The peak areas associated with digestion of human IgG at various digestion times and temperatures using a temperature stable immobilized enzyme reactor

	40°C		50°C		60°C		70°C	
	Peak area		Peak area		Peak area		Peak area	
Digest Time (minutes)	m/z - 938	m/z - 603						
15	ND	558	ND	7031	10171	120098	117598	177474
30	ND	2153	ND	9114	29754	149563	161756	179762
45	ND	1633	ND	10768	51239	139610	182325	191934
60	ND	1411	ND	10137	58822	127386	193587	188251
75	ND	1676	ND	11931	68906	116895	193231	201977
90	ND	1587	ND	13604	58927	77523	191057	169999

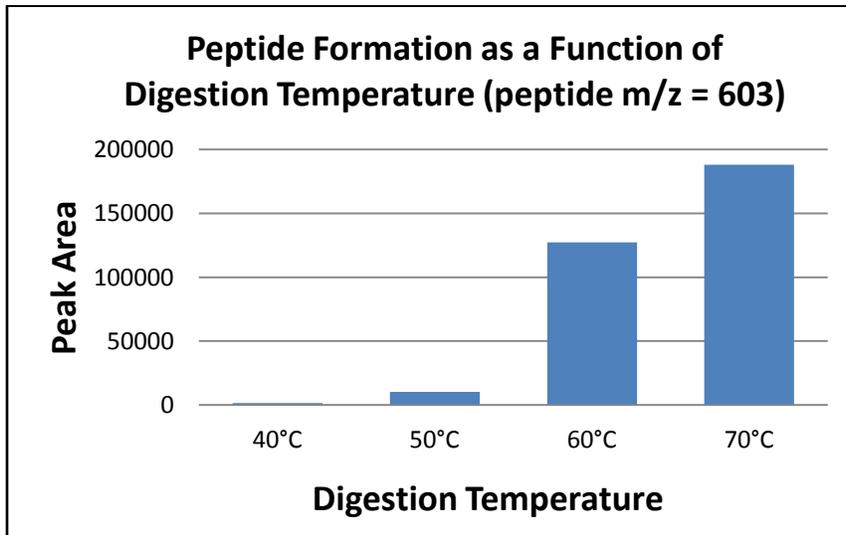
Figures 10 Peptide generation as a function of increasing digestion temperature (peptide m/z= 938, TTPPVLDSDGSFFLYSK)

Digestion Time = 60 minutes



Figures 11 Peptide generation as a function of increasing digestion temperature (peptide m/z= 603, VVSVLTVLHQDWLNGK)

Digestion Time = 60 minutes



Supplemental Data - Section 4: Solubility

Protein insolubility can be a major impediment to analysis. Insolubility can be caused by a variety of factors: salt concentrations, the presence of various organic solvents, temperature, or simply the nature of the protein itself. In some cases, this can be utilized for purification, as is seen with immunoprecipitation or pre-pelleting protocols, however, in most cases precipitation of the protein out of solution is undesirable and inhibitory to digestion.

Protein solubility can be enhanced in a number of ways. The addition of small amounts of solvents, detergents, or chaotropes can be very useful to maintaining solubility. However, as seen in our previous application note – Effect of Various Buffers Surfactants and Solvents on Trypsin Activity – many of these methods are also detrimental to trypsin digestion.

It should also be noted that digestion itself is a means to increased solubility. Most tryptic peptides, due to their smaller size, are more soluble than their parent proteins or corresponding polypeptide chains. Therefore in working to increase protein solubility under any of the conditions which may induce precipitation, we investigated a two-phase approach to maintain protein solubility: modifying the buffer composition to enhance the rate of digestion as well as provide a protein stabilizing agent.

Methods

This study employed bovine serum albumin (BSA) as a model protein. Porcine trypsin was purchased from Sigma Aldrich (St. Louis, MO). Temperature stable immobilized trypsin was made by Perfinity Biosciences (West Lafayette, IN). Tris buffered saline (TBS) was used as the default buffer and temperature was employed as the forcing factor in protein precipitation. Different concentrations of BSA were incubated at 70 °C for varying time lengths to incur varying levels of precipitation. All other chemicals used were ACS grade or higher.

Results

BSA in TBS precipitated relatively easily in any concentration about 1.25 mg/mL as seen in Figure 12A. Although this concentration is relatively high for pure protein work, it is low when considering complex matrices, which sometimes contain protein concentrations as high as 70 mg/mL. It should also be noted that as a globular protein, albumin has a relatively high solubility to begin with and many proteins that may be dealt with in a purified situation are less soluble.

First we investigated the effect of digestion on solubility. Knowing that peptides are often more soluble than their parent proteins, we expected the addition of trypsin alone would decrease the amount of precipitation observed. This was borne out in our work, as shown in Figure 12B. The addition of trypsin to the solution, even though it increased the total protein in solution by a small amount, served to dramatically reduce the amount of precipitation observed. However, concentrations of BSA greater than 2.5 mg/mL still showed precipitation after 10 minutes of incubation. This could be in part due to the denaturation of the trypsin due to the high temperature, limiting the extent to which digestion could be achieved and therefore solubility improved.

Following this we screened a wide variety of additives including organic solvents, chaotropes, surfactants, and detergents. For this screening process we fixed the concentration of BSA at the high end, 12.5mg/mL, and used a buffer that had already been improved for digestion based on the screening performed in our previous application note. We also employed temperature stable immobilized trypsin to prevent the denaturation of the trypsin from impacting our results. Samples were again run at 70 °C and 1400rpm to keep the immobilized trypsin in suspension. The results are shown in Figure 13. These results were then balanced with impacts on digestion as determined previously.

Based on the previous step's results we then employed an optimized digestion buffer which has been designed to stabilize proteins in solution. In using this buffer without trypsin we wanted to investigate it purely based on its ability to increase protein solubility without the aid of digestion seen in the previous step. At this point we found it was possible to keep BSA in solution for over 4 hours at 70 °C, even at concentrations as high as 25 mg/mL as shown in Figure 12C.

Figure 12. Precipitation of various concentrations of BSA at 70 °C for various lengths of time A: in TBS, B: in TBS with trypsin, and C: in a buffer optimized for solubility.

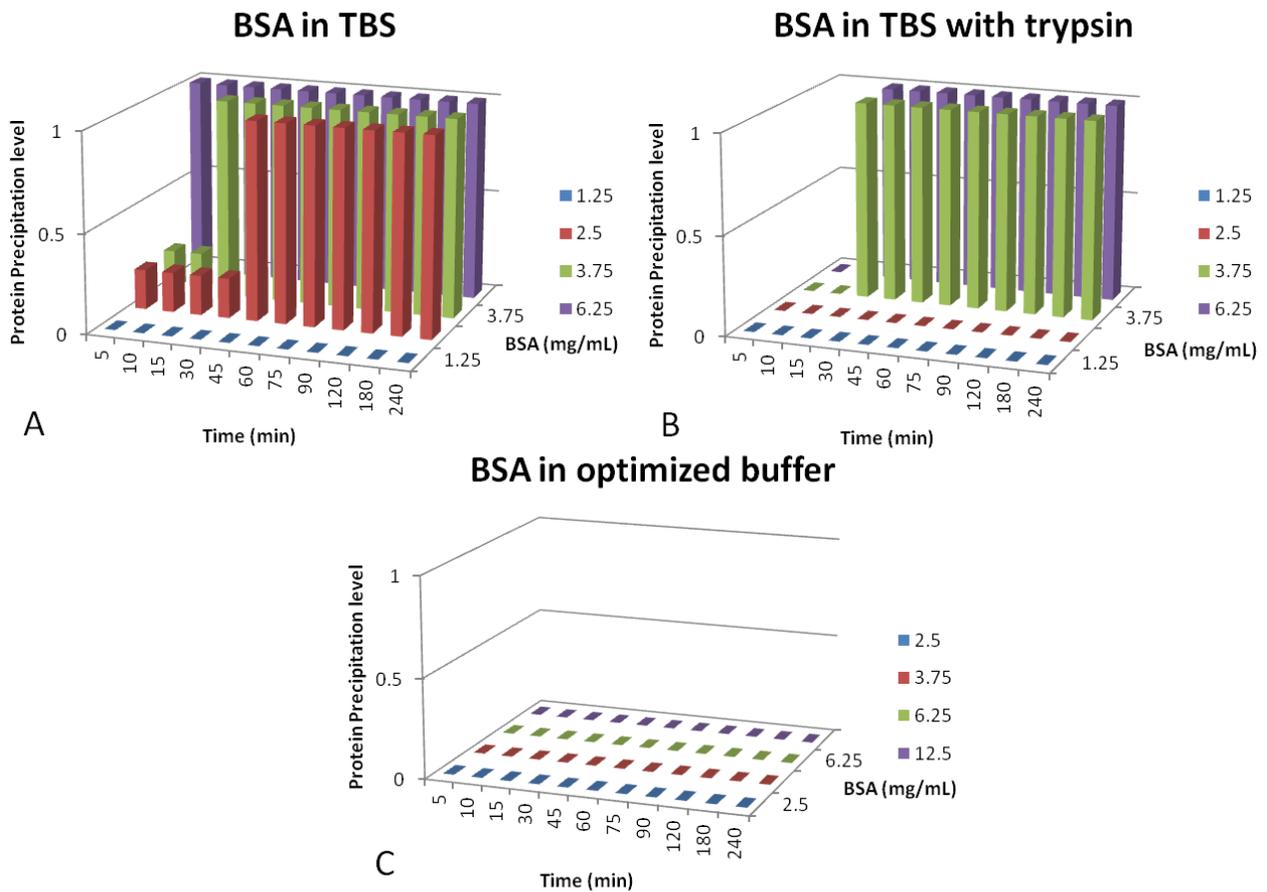
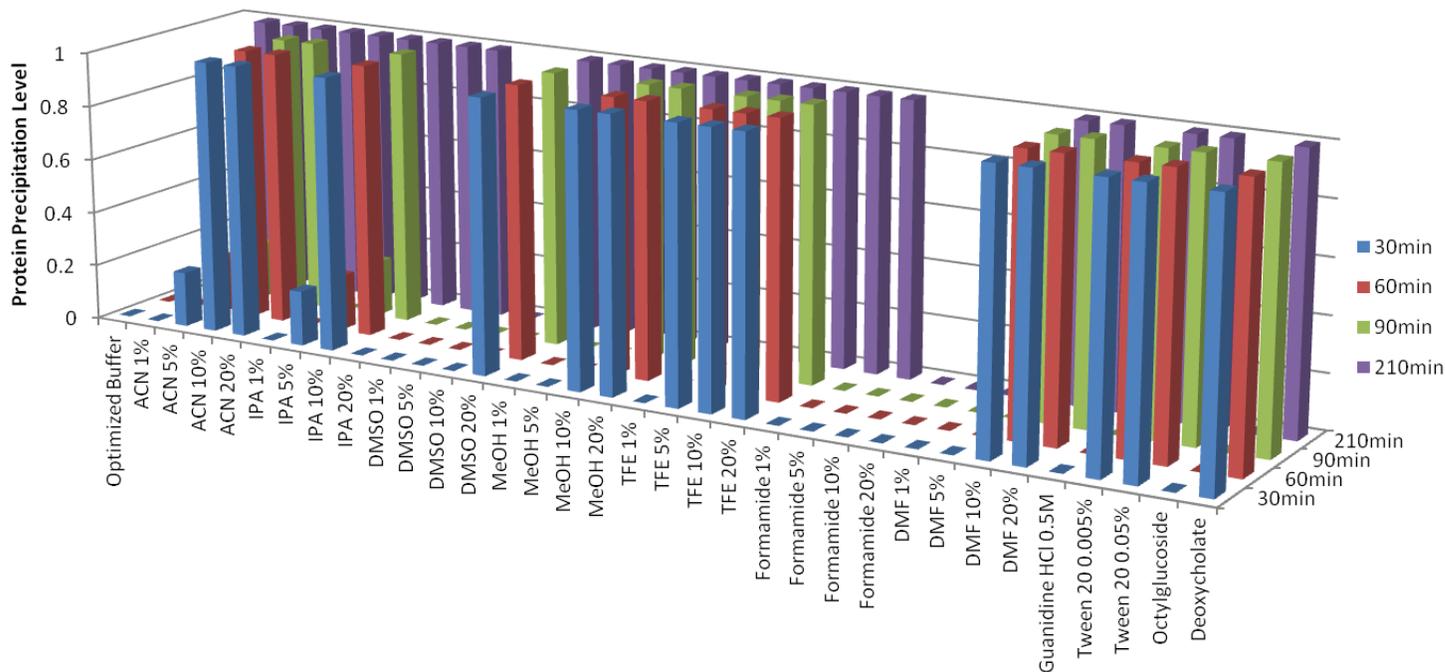


Figure 13: Precipitation of 12.5 mg/mL of BSA at 70 °C for various lengths of time in a buffer optimized for digestion and with various additives, shaken with immobilized trypsin at 1400rpm.



As a final validation of our improved buffer, we combined the digestion buffer optimized for protein stability with one optimized for digestion and employed it on samples of human plasma diluted 4 fold which also contained a thermally stable immobilized trypsin. While this only represents a final protein concentration of 17.5 mg/mL, plasma has many proteins that are far less soluble than albumin. Using this digest buffer we were able to perform digestion of 25% plasma for over 4 hours at 70 °C without precipitation.

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- [3] Kilpatrick, E.L. & Bunk D.M. (2009) *Anal. Chem.* 81, 8610-8616.