High Pressure-Accelerated Lys-C Digestion in the HUB880 Explorer: Rapid Digestion of Unreduced IgG Part III. Effects of Urea and Sodium Deoxycholate

Introduction

The positive effects of high hydrostatic pressure on digestion with Lys-C [1-6] and trypsin [1, 2, 7-9] are well established, and have been shown to result in improved sequence coverage, higher peptide intensities and significantly reduced digestion times. Additionally, the enhancing effect of pressure on the activity of several other enzymes, including chymotrypsin [10, 11], Glu-C [12], thermolysin [13], Proteinase K [14], PNGase F [15, 16], and lysozyme [17], has been reported.

Pressure, as well as heat and a variety of chemicals, can be used to denature proteins, but the pressure-perturbed proteins assume conformational forms that are different from those caused by thermal or chemical treatments [18]. Pressure-induced denaturation of substrate proteins leads to better access of enzymes to previously inaccessible, or poorly accessible, target sites. This, in turn, results in improved and accelerated enzyme activity, as long as the level of applied pressure is below the level at which the enzyme itself is denatured and inactivated. Since high hydrostatic pressure is expected to exert a strong denaturing effect on protein conformation, the effect of pressure levels up to 90,000psi (90kpsi) on Lys-C digestion was evaluated. In addition, pressure-induced denaturation, when combined with chaotropes and/or detergents that are often used in standard (atmospheric pressure) digestion reactions, can potentially lead to denaturation and inactivation of the enzyme itself. Thus, it is important to determine which reagents, and at what concentrations, are compatible with pressure-enhanced digestion protocols. Such reagents are often necessary when working with native proteins where disulfide links have not been reduced and complex tertiary structure of the substrate protein may severely limit enzyme access to target sites.

High hydrostatic pressure can accelerate protein digestion via a combination of two mechanisms. The first is the pressure-induced partial denaturation/unfolding and hydration of the target proteins, which leads to better access of the enzyme to its target sites. The second mechanism is less well understood, but relies on the increase in enzyme activity observed for some enzymes (e.g. Lys-C [3] and chymotrypsin [11]) at elevated pressure.

The benefit of high pressure incubation for enhanced Lys-C digestion of unreduced IgG, and the added benefit of reagents such as acetonitrile or N-propanol is described in separate Application Notes [4, 5]. In the current Application note we explore the effect of urea and sodium deoxycholate on pressure-enhanced digestion, in order to provide the best set of *starting conditions* for high pressure-enhanced Lys-C digestion of disulfide-intact IgG in the presence of these reagents. These conditions are likely to be similar for digestion of other hard-to-digest proteins, such as those containing hydrophobic transmembrane domains. The current application focuses on digestion at constant high pressure (not pressure cycling) in the HUB880 Explorer.

The HUB880 Explorer High Hydrostatic Pressure Generator

Pressure Biosciences' Technology uses high hydrostatic pressure to control biomolecular interactions and to accelerate certain enzymatic reactions. The HUB880 Explorer is a bench-top instrument designed for high pressure applications up to 90,000psi (620 MPa). The instrument's intensifier amplifies air pressure to generate high hydrostatic (water) pressure. The air-water intensifier has an amplification ratio of 880:1. Thus, every 1 psi of air pressure will produce approximately 880 psi of water pressure. The integrated HUB software can be used to set pressure level, rate of pressurization, time at pressure, and number of pressure cycles.

The HUB880 Explorer pressure chamber can accommodate up to 48 MicroTubes at a time, to allow batch-mode processing of multiple samples. The specially designed PCT MicroTubes are single-use sample processing containers made of a non-reactive Teflon-like material. These tubes are designed to hold 50-150µl of sample. MicroCaps of different lengths are used to displace excess air in MicroTubes. Other sample containers, that are suitable for larger sample volumes up to 1.4ml, are also available.

Materials and Methods

Lysyl Endopeptidase enzyme (Lys-C) was purchased from Wako Chemicals USA, Inc. (mass spectrometry grade, Cat# 125-05061). Model proteins; equine myoglobin and human gamma globulin (IgG) were from Sigma. Protein stocks were prepared and stored frozen in aliquots. IgG was digested without reducing disulfide bonds (myoglobin does not contain disulfide bonds).

All digests, including controls at ambient pressure, were incubated in PCT microTubes at 42^0 C, unless otherwise indicated. All high pressure IgG digestions were carried out using a single constant pressure hold, rather than repeated pressure cycling. High pressure incubations were carried out in the HUB880 Explorer, using pump mode to reach the set pressure, with a 30 minute hold at the set pressure. Enzyme to substrate ratio was 1:400 for all IgG digests, unless indicated otherwise.
Digested and control samples were reduced with DTT and denatured by boiling in Laemmli buffer, prior to gel electrophoresis. Digestion was assayed by loss of intact protein bands on SDS-PAGE.

Lys-C digestion of myoglobin under different pressure conditions was followed using OD_{405} assay, for quantification of the effect of pressure on model protein digestion, which takes into account the effect of pressure on the enzyme as well as the effect on the substrate protein. Myoglobin (0.5 mg/ml) was incubated in 100mM Ambic supplemented with the indicated solvent, at 42^0 C for 15 minutes, at the indicated pressure in the HUB880 Explorer. Lys-C was added at a 1:500 enzyme-to-substrate ratio. For comparison, control reactions were incubated without enzyme at both Atm and 85kpsi. OD_{405} was measured just prior to, and directly after, high pressure incubation. Digestion of the myoglobin protein results in a reduction in absorbance measured at OD_{405}. The change in OD of samples incubated at different pressures was used to generate linear slopes to estimate protein digestion rate and enzyme activity. The values in Figure 5B are presented as inverse slopes, so that decrease in OD_{405} is translated into increase in reaction rate.

Results and Discussion

The benefit of pressure cycling for Lys-C protein digestion is well established. Digestion of the difficult-to-digest model protein myoglobin is described in an earlier Application Note [3]. Accelerated digestion of disulfide-intact IgG by Lys-C with trypsin [9C] has also been demonstrated. Previous data demonstrated that significant improvement in Lys-C digestion is achieved at 45kpsi, the highest pressure that can be reached in the 2320EXT unit. In addition, the specificity of Lys-C digestion at 45kpsi has been examined and no significant increases in semi-specific cleavage or miscleavage have been observed [19]. Recent results have demonstrated the benefit of pressures above 45kpsi for improved Lys-C digestion of unreduced IgG [4, 5].

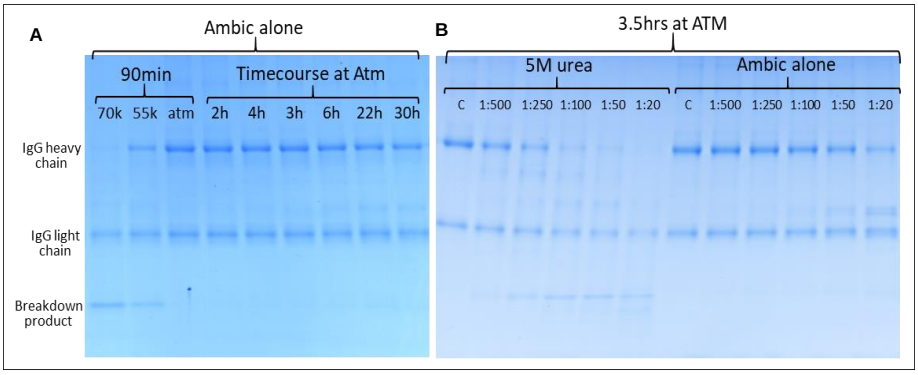

Figure 1. Lys-C digestion of human IgG at atmospheric pressure requires high enzyme concentration and long reaction time. Digestion is significantly accelerated by high pressure, or by addition of urea. A. Unreduced IgG was incubated with Lys-C (E/S 1:1000) at 42^0 C for the indicated time. Digests were incubated either at ambient pressure (Atm), or at 70kpsi or 55kpsi. Digestion at ambient pressure is very poor, even after 30 hours. By contrast, much digestion is evident at both 55 and 70kpsi in as little as 90 mins. **B**. Unreduced IgG was incubated in 100mM Ambic +/- 5M urea, with Lys-C at the indicated E/S ratio, for 3.5 hours at 37^0 C. Controls were incubated without enzyme (C). Significant improvement in IgG digestion occurs when reactions are supplemented with urea. The combined effect of urea and pressure is shown in Figures 2, 3 and 5.

IgG is a large, difficult-to-digest protein, made up of four polypeptide chains (2 heavy chains and 2 light chains) held together by several disulfide bonds. The current work focuses on the effect of higher pressure levels, up to 90kpsi, on Lys-C digestion of unreduced IgG in the presence of urea, a chaotrope often used to unfold proteins for improved digestion, and sodium deoxycholate (DOX), a detergent sometimes used in place of urea.

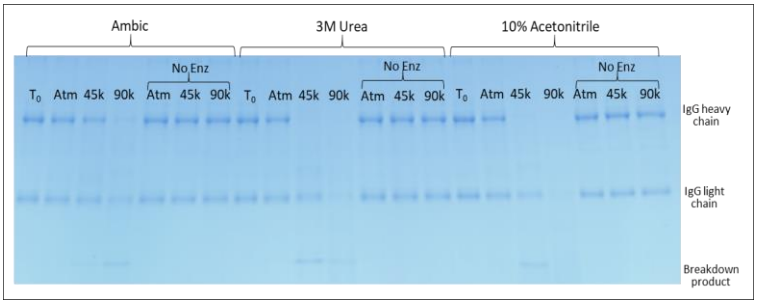

Figure 2. Accelerated Digestion at High Pressure: Effect of pressure level and buffer composition. Reactions were incubated at the indicated pressure for 30mins, in 100mM Ambic supplemented with the indicated reagent. Untreated controls (T_0) and those incubated without enzyme, are shown for comparison. In all three reagents, digestion is poor at Atm and is most complete at 90kpsi. Completeness of digestion appears similar in both urea and acetonitrile, and is significantly increased compared to reactions in Ambic alone.

Digestions were carried out at high pressure in the HUB880 Explorer, using a single constant pressure hold, rather than repeated pressure cycling. The benefit of high pressure on Lys-C digestion of unreduced IgG is shown in **Figure 1**. Digestion in ammonium bicarbonate (Ambic) alone is slow at atmospheric pressure, and requires a high enzyme-to-substrate ratio. The reaction can be accelerated by adding urea to the buffer, or by carrying out the reactions at high pressure. The benefit of combining urea and high pressure digestion is described below.

Previous results had shown that high pressure-accelerated Lys-C digestion of IgG could be significantly enhanced by adding 10% acetonitrile to the reactions [5]. To determine whether urea could have a similar effect we compared digestion in Ambic alone, or in 10% acetonitrile, to 3M urea, at three pressure levels (Atm, 45kpsi, and 90kpsi). The results, shown in **Figure 2** demonstrate that urea has a similar effect to acetonitrile on the pressure-accelerated digestion of IgG. The reaction is not significantly accelerated at atmospheric pressure, but at 45kpsi and 90kpsi, a significant benefit is observed. In the presence of 3M urea, digestion at 45kpsi appears to be as effective as digestion at 90kpsi in buffer alone. To further explore the synergy of urea and high pressure, several concentrations were tested at different pressure levels. The results, shown in **Figure 3,** indicate that overall, the most complete digestion of both the heavy and light chains, is observed in 3M urea at 70kpsi, although digestion at 45 and 55kpsi is also significantly improved. The small breakdown product (near the bottom of the gel), is quite difficult to digest, but can be effectively digested either at 70kpsi in 5M urea, or in 3-5M urea at 90kpsi.

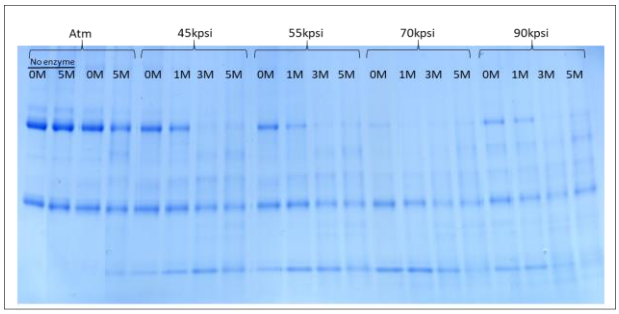

Figure 3. Accelerated Digestion at High Pressure: Synergy of pressure level and urea concentration. Reactions were prepared in 100mM Ambic supplemented with the indicated concentration of urea. Digests were incubated for 30 mins at the indicated pressures. Best digestion is observed in 3M urea at all high pressure levels. Best overall digestion is at 70kpsi in 3M urea.

These results suggest that the optimal pressure for digestion will vary, depending on the urea concentration present in the sample. However, the addition of 3M urea does not appear to result in loss of enzyme activity, even at the highest pressures tested.

One drawback of using urea to unfold substrate proteins is the carbamylation reaction. Urea-induced carbamylation is significantly increased at higher temperatures; to minimize protein carbamylation, reactions that contain urea should not be exposed to temperatures significantly above ~37^0 C. Since we have previously demonstrated that Lys-C digestion of unreduced IgG at high pressure is significantly accelerated when reactions are carried out at temperatures up to ~55^0 C [4,5], we tested the effect of replacing urea with sodium deoxycholate (Dox), an ionic detergent that has previously been shown to be effective in high pressure-accelerated protein digestion applications [9G]. Results presented in **Figure 4** suggest that addition of low concentrations of Dox, between 0.1% and 1.0%, can improve digestion of IgG at lower pressure levels (i.e., at 45 and 55kpsi), compared to digestion in Ambic alone. However, at higher pressures, better digestion is observed at 0.1% Dox, the lowest concentration tested. Overall, best digestion was observed at 70kpsi in 0.1% Dox. Increasing pressure to 85kpsi does not improve digestion, and resulted in slightly less effective digestion in some cases.

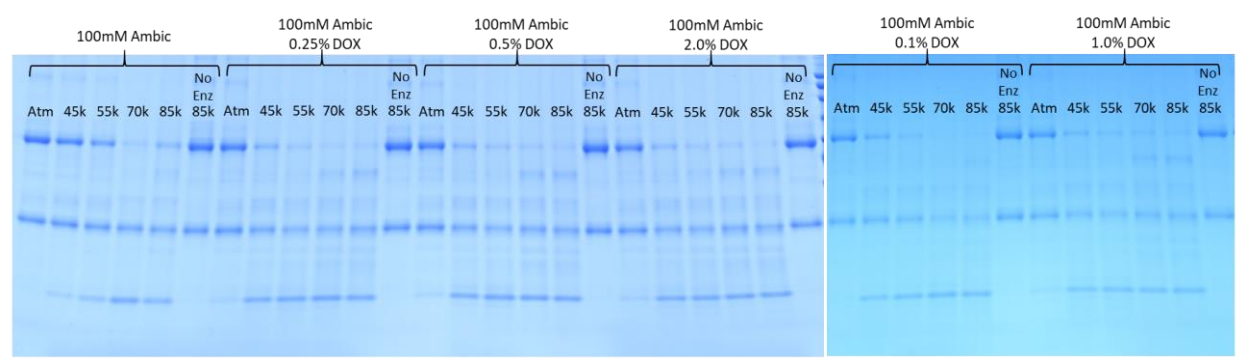

Figure 4. Accelerated Digestion at High Pressure: Synergy of pressure level and Sodium deoxycholate (Dox) concentration. Reactions were incubated at 42^0 C, for 30 mins, in 100mM Ambic with the indicated concentration of Dox, at the indicated pressure. Lys-C was added at 1:400 E/S ratio. Untreated controls incubated at 85kpsi (no enzyme) are shown for comparison. Addition of 0.1% Dox slightly improves digestion above what is seen in Ambic alone. Overall best digestion is at 70kpsi in Ambic with 0.1% Dox.

Myoglobin digestion results in a drop in OD_{405} over time. The slope of the signal drop can be used as an estimate of the reaction rate (for clarity, Figure 5B presents the inverse slope, so that increased digestion is seen as an increase in signal). In agreement with data presented in Figures 1-4 above (and in previous Application notes [4, 5]), the results in **Figure 5** show a pressure level-dependent increase in digestion rate. At ambient pressure (Atm), digestion in all buffer formulations is very slow. In Ambic alone, digestion rate increases steadily as pressure is increased from 45kpsi to 70kpsi, and is highest at 70-85kpsi. Incubating the myoglobin at high pressure without enzyme, results in a very slight, pressure-dependent effect on OD_{405}, likely due to change in optical density associated with myoglobin unfolding under pressure. This unfolding is likely to make the myoglobin a better substrate for lys-C, and contributes to the increase in Lys-C digestion activity associated with high pressure. Adding 1M urea significantly increases digestion at the lower pressure levels (45 and 55 kpsi), but the benefit at higher pressures is less pronounced. At 70 and 85kpsi, digestion in 1M urea is only slightly better than in Ambic alone, suggesting that for applications where addition of urea is not desirable, digestion in Ambic alone at 70kpsi can obviate the need for urea. In the presence of 3M urea, digestion rate appears to be maximal at 45kpsi, and is not further improved by increasing pressure. Therefore, if 3M urea is acceptable, digestion can be carried out at lower pressures, such as are achievable in the 2320EXT or HUB440 Explorer Barocycler models. Note that even in 3M urea, digestion at Atm is barely detectable during the 15minute incubation time, while at 45kpsi, the myoglobin parent protein band is almost completely digested (Figure 5C). The myoglobin results in Figure 5 agree well with the IgG digests in Figures 1-3, suggesting that urea at concentrations in the 1-3M range can be used in conjunction with high pressure, for rapid Lys-C digestion of unreduced IgG.

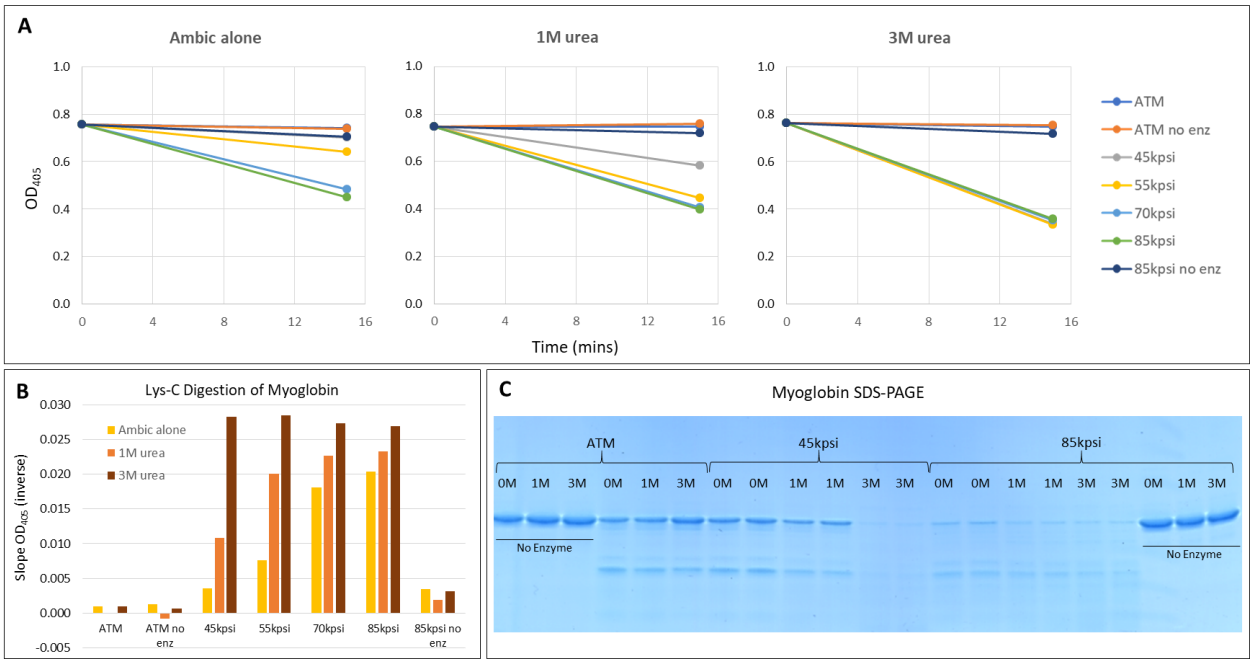

Figure 5. Lys-C activity assay with myoglobin substrate: Effect of high pressure level and urea concentration on digestion rate. **A**. OD_{405} was measured at time zero, and after 15 minutes at the indicated pressure. All reactions were carried out in 100mM Ambic supplemented with the indicated concentration of urea. Control reactions incubated without enzyme at Atm or at 85kpsi are also shown. A drop in OD reading is indicative of protein digestion. All values are average of triplicates. **B.** Data generated in **A** are presented as inverse slope, so that increased digestion is seen as an increase in signal. **C.** Myoglobin digestion visualized by SDS-PAGE. Appearance of myoglobin after 15 minute digestion at the indicated pressure in Ambic alone (0M urea) or supplemented with 1M or 3M urea. Protein digestion is seen as loss of the intact parent protein band, and the appearance of a small breakdown product near the bottom of the gel.

Conclusions

Here we examined the pressure-enhanced Lys-C digestion of disulfide-intact human IgG. Digestion of this large (~250kDa) disulfide-crosslinked protein is very slow under normal atmospheric pressure conditions, even in the presence of 3-5M urea. As was recently shown with Trypsin [9F], the reaction rate can be significantly accelerated by incubating at high pressure in the HUB880 Explorer. Previous results [3], have indicated that Lys-C digestions can be accelerated by performing the reactions at 45kpsi with pressure cycling. More recently, we demonstrate that Lys-C digestion of unreduced IgG, as well as other proteins, can be significantly accelerated when incubated at constant high pressure. For reactions in mild buffer (100mM Ambic), the optimal pressure appears to be 70kpsi, at temperatures in the 37^0 C to 55^0 C range. At higher temperatures, slightly lower pressure levels (e.g., ~45-55kpsi) work better [4]. In addition, the benefit of adding a small amount of organic solvent to the high pressure digestions has also been demonstrated [5].

In the present Application Note we explore the effects of urea and sodium deoxycholate on high pressure-accelerated digestion of IgG by Lys-C enzyme. In agreement with previous results [4, 5], in the absence of added reagents (i.e., in Ambic alone), best digestion is seen at ~70kpsi. Addition of 1M or 3M urea is shown to significantly enhance the benefit of high pressure. The addition of Dox is shown to be compatible with high pressure digestion and can accelerate digestion at the lower pressure levels (45-55kpsi). Overall, the best condition for lys-C digestion of unreduced IgG appears to be 1M urea at 70kpsi, although excellent digestion can also be achieved in the absence of urea for applications where urea-induced carbamylation is undesirable.

The table below summarizes some of the recommended high pressure conditions for Lys-C digestion of unreduced IgG in the HUB880 Explorer (for more information on the effect of temperatures and solvents on Lys-C digestion, see two companion Application notes [4, 5] from PBI). These guidelines are a starting point for further optimization by individual users to generate pressure-enhanced protocols for their specific needs and sample types.

Suggested Starting Conditions for Pressure-accelerated Lys-C Digestion of Unreduced Human IgG in the HUB880 Explorer.

Buffer composition	Temperature	Suggested Pressure	Notes
50-100mM ammonium bicarbonate alone	37-45^0 C	70kpsi	Incubation time of 1-2 hours is suggested. Optimal incubation time for complete digestion will require optimization by the user. Performing digestions at 37^0 C or less may require longer digestion times.
	50-52^0 C	45-60kpsi (~55kpsi is recommended)	
50-100mM ammonium bicarbonate with 1M urea	35-37^0 C	70kpsi (up to 90kpsi is compatible)	Incubation time of 0.5-2 hours is suggested. Optimal incubation time for complete digestion will require optimization by the user. To minimize carbamylation due to urea, keep reactions at 35-37^0 C. Performing digestions at temps below 37^0 C may require longer digestion times. If carbamylation is not a concern, increase to 42^0 C. Increasing urea concentration above 3M is compatible with high pressure digestion, but may require optimization of pressure level by the user.
50-100mM ammonium bicarbonate with 3M urea	35-37^0 C	45kpsi (up to 70kpsi is compatible)	
50-100mM ammonium bicarbonate with 0.1% sodium deoxycholate (Dox)	42^0 C Higher temps have not been tested.	70kpsi	Incubation time of 1-2 hours is suggested. Optimal incubation time for complete digestion will require optimization by the user. Increasing Dox concentration up to 1% is compatible with high pressure digestion, but may require optimization of pressure level by the user.
50-100mM ammonium bicarbonate **with 10% N-Propanol**	42^0 C Higher temps have not been tested.	45-85kpsi	Incubation time of 0.5-2 hours is suggested. Optimal incubation time for complete digestion will require optimization by the user.
50-100mM ammonium bicarbonate **with 10% Acetonitrile**	42-55^0 C	70-90kpsi	Incubation time of 0.5-2 hours is suggested. Optimal incubation time for complete digestion will require optimization by the user. Performing digestions at 37^0 C or less may require longer digestion times.
50-100mM ammonium bicarbonate **with 20% Acetonitrile**	42^0 C Higher temps have not been tested.	55-70kpsi	Incubation time of 0.5-2 hours is suggested. Optimal incubation time for complete digestion will require optimization by the user.
50-100mM ammonium bicarbonate **with 10% Methanol**	42^0 C Higher temps have not been tested.	70kpsi	Digests containing 10% methanol can be treated in the same way as in Ambic alone. The effect of higher methanol concentration has not been evaluated.

* Digests at 45kpsi or lower, can be performed in the 2320EXT or the HUB440 Explorer, as well as in the HUB880 Explorer.

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