High Pressure-Accelerated Lys-C Digestion in the HUB880 Explorer: Rapid Digestion of Unreduced IgG Part II. Effect of Acetonitrile, N-Propanol, and Methanol

Introduction

The positive effects of high hydrostatic pressure on digestion with Lys-C [1-5] and trypsin [1, 2, 6-8] 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 [9, 10], Glu-C [11], thermolysin [12], Proteinase K [13], PNGase F [14, 15], and lysozyme [16], 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 [17]. 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 [10]) 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 urea and sodium deoxycholate, is described in separate Application Notes [4, 19]. In the current Application Note we explore the effect of several organic solvents 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.
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 in order 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 with trypsin/Lys-C [8C] has also been demonstrated. The specificity of Lys-C digestion at 45kpsi has been examined and no significant increases in semi-specific cleavage or miscleavage have been observed [18].
The current application focuses on Lys-C digestion of disulfide-intact human IgG at pressures significantly above those that can be achieved in the 2320EXT. Trypsin digestion of the unreduced (disulfide-intact) protein has been shown to be greatly improved by incubating the reactions in the HUB880 Explorer at 80kpsi [8F].

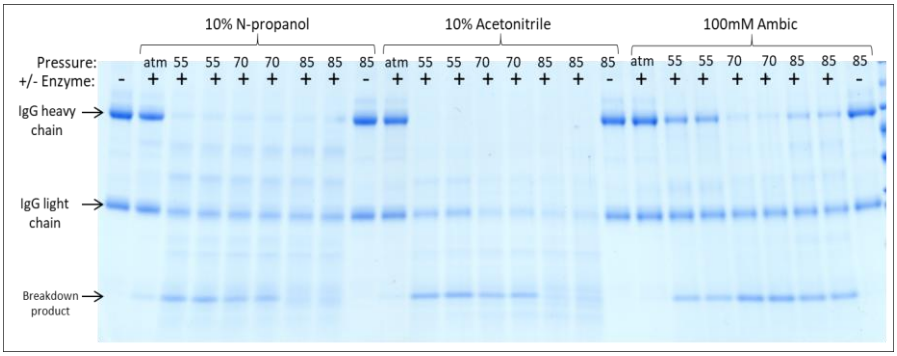

Figure 1. High pressure accelerated digestion by Lys-C can be further improved by adding organic solvents such as acetonitrile or N-propanol. All reactions were incubated for 30 mins, at 42^0 C, with Lys-C at 1:400 E/S ratio [controls incubated at 85kpsi without enzyme (-) are also shown], at the indicated pressure (kpsi). Reactions were prepared either in 100mM Ambic alone, or supplemented with 10% acetonitrile or 10% N-propanol. The effect of reaction pressure and buffer composition on the digestion of IgG is assayed by loss of the intact protein bands on SDS-PAGE. The positive effect of the two organic solvents on high pressure digestion of unreduced IgG is readily apparent. Little, if any, digestion is observed in the samples incubated at Atm for 30 mins, with or without solvents. Digestion is significantly enhanced at high pressure, and is almost complete in 30 mins at 85kpsi in 10% acetonitrile. Untreated control IgG (-) is shown for comparison.

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 benefit of high pressure incubation on digestion of unreduced IgG by Lys-C in ammonium bicarbonate (Ambic) buffer is described in another Application Note [4]. The current work focuses on the effect of pressure levels up to 90kpsi, on Lys-C digestion of unreduced IgG in the presence of organic solvents (acetonitrile, N-propanol, and methanol).

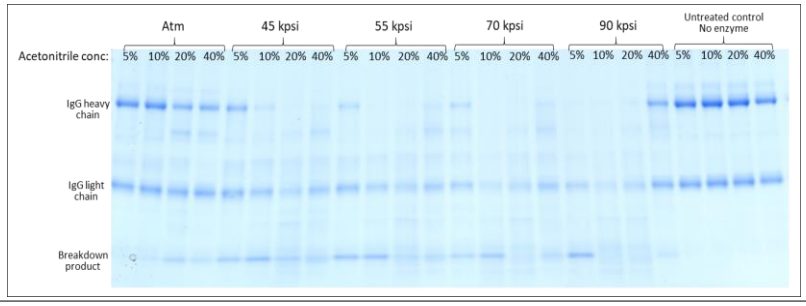

Figure 2. Accelerated Digestion at High Pressure: Effect of acetonitrile concentration and pressure level. Reactions were incubated for 30 mins in 100mM Ambic supplemented with the indicated concentration of acetonitrile, at the indicated pressure. Untreated controls (no enzyme) are shown for comparison. Best digestion is observed either at 55kpsi in 20% Acn, or at 90kpsi in 10% Acn. In 40% Acn, the combined effect of high pressure and high solvent concentration result poor digestion, especially at 90kpsi.

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 Ambic is slow at atmospheric pressure, and is accelerated with increasing pressure level, showing optimal digestion at 70kpsi. Incubation at higher pressure (85kpsi) results in less effective digestion, likely due to the effect of pressure on enzyme structure. In the presence of 10% N-propanol, digestion is enhanced at all high pressure levels. In addition, the solvent appears to protect the enzyme from high pressure inactivation, as digestion at 85kpsi does not show the drop-off that is observed in Ambic alone. Results in 10% acetonitrile (Acn) are similar to N-propanol, with even better digestion as indicated by the more complete loss of protein bands from the gel. To further explore the effect of acetonitrile on digestion of IgG,

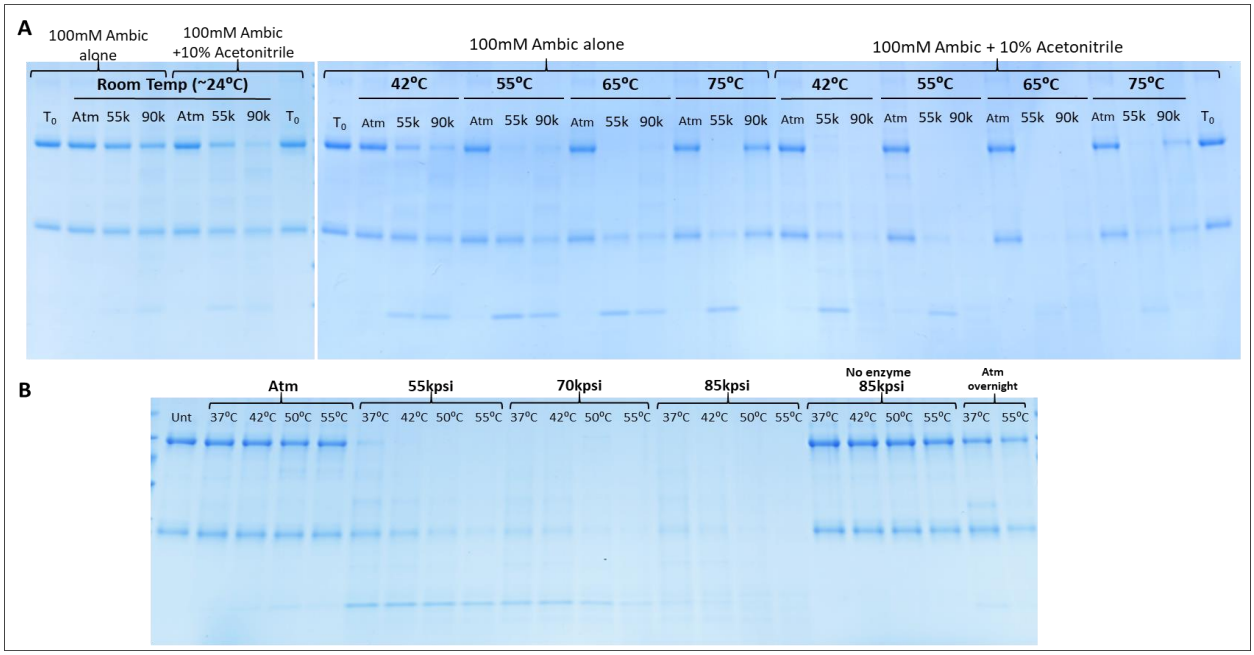

Figure 3. Accelerated Digestion at High Pressure: Synergy of temperature and pressure level. A. Reactions were incubated for 30 mins in 100mM ammonium bicarbonate, with or without 10% acetonitrile, at the indicated temperature and pressure. Untreated controls (T_0) are shown for comparison. Best digestion is observed either at 90kpsi/55^0 C, or at 55kpsi/65^0 C, and is further enhanced in the presence of 10% Acn. **B.** Reactions were incubated for 30 mins in 100mM ammonium bicarbonate /10% Acetonitrile, at the indicated temperature and pressure. Untreated control (Unt) is shown for comparison. In this moderate temperature range (37^0 C to 55^0 C), higher pressure results in more complete digestion and best results are achieved at 85kpsi at all four temperatures. The benefit of increasing temperature from 37^0 C to 55^0 C is seen at

solvent concentrations between 5% and 40% were tested at different pressure levels. The results, shown in **Figure 2,** demonstrate that Acn concentration in the 10-20% range is optimal for high pressure Lys-C digestion

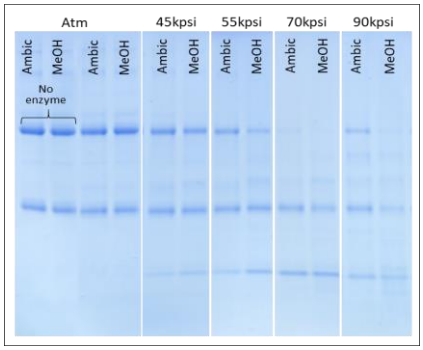

Figure 4. High pressure-accelerated digestion in buffer supplemented with 10% methanol. Reactions were incubated at 42^0 C, for 30 mins, in 100mM Ambic alone, or with 10% MeOH, at the indicated pressure. Untreated controls (no enzyme) are shown for comparison. Best digestion is observed at 70kpsi, with or without MeOH, or at 90kpsi in 10% MeOH.

of IgG. The synergy of the solvent concentration and pressure level is demonstrated by the inverse relationship; at 45-55kpsi, digests proceed more quickly in 20% Acn than in 10%, however at 70-90kpsi, better digestion is seen in 10%, rather than 20%, Acn. While the presence of 40% Acn is compatible with pressure-accelerated digestion at pressure levels up to 70kpsi, the combined effect of 90kpsi pressure and the high solvent concentration, result in very poor digestion at this pressure level.

An example of the synergy of temperature and pressure level is demonstrated in **Figure 3**. At moderate temperatures (below ~55^0 C), digestion at 90kpsi is more effective than at 55kpsi. At higher temperatures (above ~55^0 C), the combined effect of heat and high pressure on the enzyme structure results in less effective digestion at the higher pressure. **Note that at all temperatures examined, digestion at 55kpsi is significantly more effective than digestion at ambient pressure.**

As seen in Figure 1, adding 10% acetonitrile to high pressure reactions results in more complete digestion of the IgG light chain, than is achieved in samples without Acn. Overall, the most complete digestion of both the heavy and light chains, as well as of the small breakdown product, is observed in 10% Acn, either at 90kpsi/55^0 C, or at 55kpsi/65^0 C. These results suggest that the optimal pressure for digestion will vary, depending on the temperature condition being used.

Based on the results shown in Figure 3A, which suggest a significant difference in digestion efficiency between 55kpsi and 90kpsi, mid-range pressures of 70kpsi and 85kpsi were compared to 55kpsi and Atm. In addition, since Lys-C digestions are not usually carried out at high temperatures in the 65-75^0 C range, a narrow temperature range that is more commonly used for lys-C digestion (37-55^0 C) was examined. The results shown in **Figure 3B** highlight the benefit of high pressure-accelerated digestion, and suggest that in this temperature range, maximal digestion is observed at 85kpsi. Digestion at a lower and slightly less optimal pressure (e.g., 55kpsi) can be significantly enhanced by increasing the temperature from 37^0 C to 55^0 C, further highlighting the synergy of pressure and temperature for enhancing digestion.

In order to examine the effect of high pressure on enzyme reaction rate in an assay that takes into account the effect of pressure on both the enzyme and substrate proteins, we used myoglobin as a substrate protein in Lys-C digests under various pressure and solvent conditions. Digestion of myoglobin, a dark red/brown heme-containing protein, can be followed by measuring optical density at 405nm (OD_{405}). 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, the results in **Figure 5** show a pressure-dependent increase in digestion rate when the reactions are carried out at high pressure. The effect of adding 10% N-propanol or 10% methanol to the reactions also agrees with the results obtained with IgG, suggesting that N-propanol has a significant positive effect on pressure-accelerated Lys-C digestion, while methanol has little overall effect and reactions supplemented with methanol proceed essentially at the same rate as reactions in Ambic alone. As seen with unreduced IgG, the most rapid Lys-C digestion occurs at 70-85kpsi in Ambic alone (or supplemented with 10% methanol). However, by adding 10% N-propanol, very significant increases in reaction rate at 45-55kpsi can be achieved. 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.

The effect of adding methanol, a commonly used HPLC solvent, to the high pressure digest reactions is presented in **Figure 4.** Unlike what is observed with N-propanol or acetonitrile, adding methanol appears to have relatively little effect on pressure-accelerated Lys-C digestion, although a slight benefit is seen at 55 and 70kpsi. However, the data do suggest that at the highest pressure level tested (90kpsi), adding 10% MeOH

may have a noticeable benefit for digestion, possibly by protecting the enzyme from pressure-induced denaturation. This putative mitigating effect of methanol on very high pressure-induced reduction in Lys-C activity could be relevant for applications where recalcitrant proteins may require very high pressure levels (above 70kpsi) to achieve sufficient unfolding for adequate Lys-C digestion.

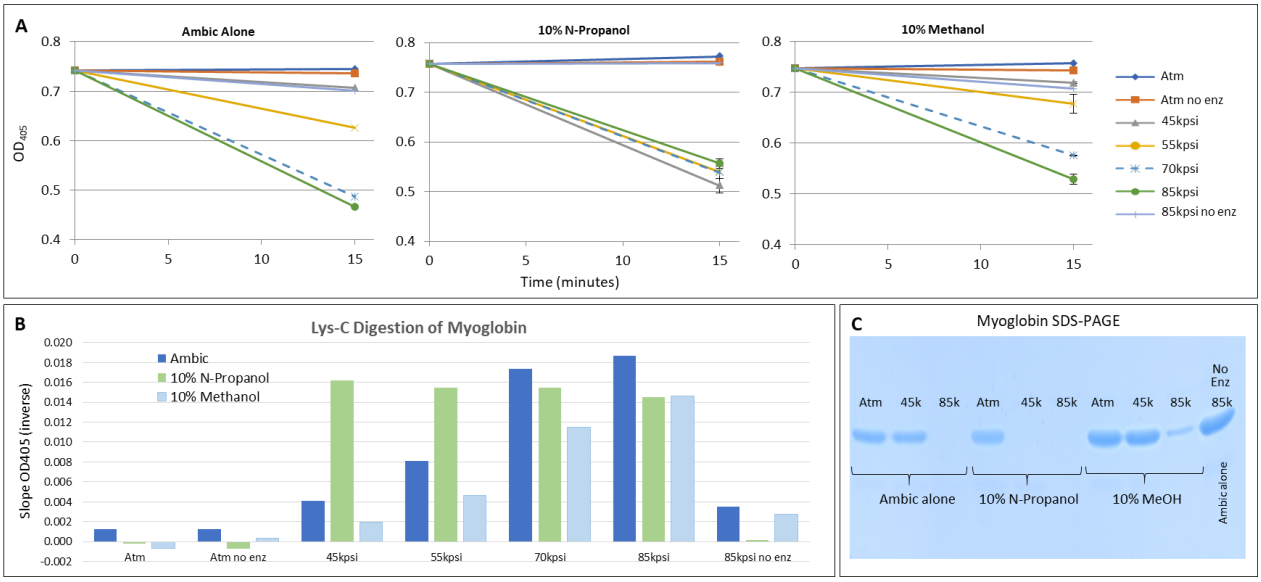

Figure 5. Lys-C activity assay with myoglobin substrate: Effect of high pressure level and buffer composition on digestion rate. **A**. OD_{405} was measured at time zero, and after 15 minutes at the indicated pressure. All reactions were carried out in 100mM Ambic supplemented with the indicated solvent. 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 (error bars indicated standard deviation). **B.** Results from multiple independent experiments (such as the examples shown in **A**), were pooled. Data 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 and buffer. Protein digestion is seen as loss of the intact parent protein band.

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. As was recently shown with Trypsin [8F], by performing the reactions under high pressure in the HUB880 Explorer, the reaction rate can be significantly accelerated. 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 the present Application Note we explore the effect of several organic solvents on high pressure-accelerated digestion of IgG by Lys-C enzyme. In the absence of added solvents (i.e., in Ambic alone), best digestion is seen at ~70kpsi. Addition of 10-20% acetonitrile is shown to significantly enhance the benefit of high pressure, with maximal digestion rate occurring at 55-70kpsi. The effect of adding 10% N-propanol, while not as beneficial as acetonitrile, is nevertheless very significant, resulting in rapid pressure-accelerated digestion of unreduced IgG. In contrast, 10% methanol appears to have relatively little effect on the digestions at all pressures tested. Additional Application Notes explore the effect of adding or omitting certain chaotropes on high pressure Lys-C digestion of disulfide-intact IgG [4, 19].

The table below summarizes some of the recommended high pressure conditions for Lys-C digestion of unreduced IgG in the HUB880 Explorer. 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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Part III. Effect of Urea and Sodium Deoxycholate (document in preparation)