High Pressure-Accelerated Digestion of Unreduced IgG by Lys-C and Trypsin

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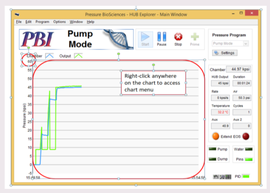
Introduction

Efficient and rapid proteolytic digestion of disulfide-intact monoclonal antibodies could significantly improve overall routine workflows in protein characterization and biopharmaceutical quality control. Conventional approaches use extensive denaturation by guanidine and/or urea, followed by overnight incubation with high concentrations of enzyme(s). Such protocols frequently produce sporadic miscleavages and other artifacts of long protocols, as deamidation, methionine oxidation, and disulfide scrambling. Pressure-induced protein denaturation leads to better access of enzymes to previously inaccessible, or poorly accessible, sites, resulting in rapid and reproducible digestion. For proteins such as unreduced IgG, higher pressure levels may be necessary for effective unfolding while keeping the disulfide bridges intact. Since pressure exerts a strong denaturing effect, IgG digestion by Lys-C or trypsin at pressures up to 90,000psi was evaluated. In addition, pressure is known to be synergistic with chaotropes, organic solvents, or detergents in denaturing not only substrate proteins, but also affecting the enzyme activity. Thus, it is important to determine which reagents, and concentrations, are compatible with pressure-enhanced digestion of difficult proteins.

The HUB880 Explorer High Hydrostatic Pressure Generator

Pressure Biosciences' HUB880 Explorer is a bench-top instrument capable of incubating samples at pressure up to 90,000 psi (620 MPa) and temperature up to 100°C. The HUB880 Explorer pressure chamber can accommodate up to 48 MicroTubes at a time, to allow batch-mode processing of multiple samples.



HUB880 Explorer Pump Mode Software

PCT MicroTubes

Materials and Methods

Trypsin, myoglobin, and human gamma globulin (IgG) were from Sigma. Lysyl Endopeptidase enzyme (Lys-C) was from Wako Chemicals USA, Inc. Digests were carried out in ammonium bicarbonate buffer (AmBic), with or without the indicated reagents, using 100ul of reaction per MicroTube, with the indicated enzyme-to-substrate ratio (w/w). All digests were carried out using disulfide-intact (i.e., unreduced) IgG. Digestion was assayed by loss of intact protein bands on SDS-PAGE. 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, unless indicated otherwise.

Trypsin activity was measured using the chromogenic substrate, N_{α}-Benzoyl-D, L-arginine 4-nitroanilide hydrochloride (BAPNA). OD_{405} was measured at 1 second intervals for 3 minutes. The slope of the OD_{405} increase was used as an estimate of enzyme activity.

Lys-C activity under different pressure conditions was followed using myoglobin as the substrate (0.5 mg/ml), in 100mM AmBic buffer with or without Lys-C (1:500 E/S) at 42°C for 15 minutes at the indicated pressure. OD_{405} was measured just prior to, and directly after, high pressure incubation. Digestion of the myoglobin protein results in a reduction in absorbance at OD_{405}. This assay allows 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.

Results Part I: Trypsin Digestion at High Pressure

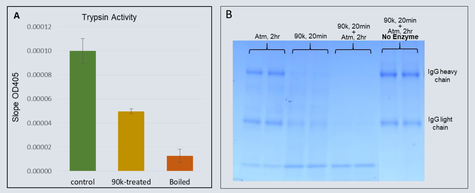


Figure 1. Trypsin exposed to a pressure of 90kpsi retains significant enzymatic activity. **A**. Trypsin was pretreated for 20min at 90kpsi or at ambient pressure. Activity after pre-treatment was assayed at ambient pressure, by BAPNA assay. For comparison, activity of trypsin inactivated by boiling (5 min at 100°C) is also shown. The assay demonstrates that trypsin either retains or regains much of its activity (~50%) after 20min at 90kpsi. **B**. Unreduced human IgG was digested with trypsin (1:50 E/S) in 50mM AmBic buffer at 50°C (in duplicate). To examine trypsin activity at high pressure, reactions were incubated at 90kpsi for 20 min. Some reactions were then incubated at ambient pressure for a further 2 hours. Control digests were held at ambient pressure. Negative controls were incubated without enzyme. The results demonstrate that unreduced IgG digestion is accelerated at 90kpsi, showing significant digestion of both the heavy and light chains in just 20 minutes, compared with relatively little digestion after 2 hours at atm. In agreement with the data in Panel A, trypsin digestion continues once the 90kpsi-treated reactions are transferred to ambient pressure, confirming that the enzyme remains active.

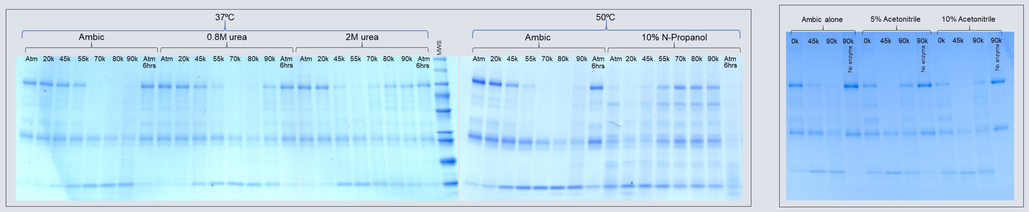


Figure 2. Effect of pressure level and buffer formulation on trypsin digestion of unreduced IgG. A. Digests were incubated for 30 minute with trypsin (1:50 E/S), in 50mM AmBic buffer supplemented with the indicated reagent, at the indicated temperature and pressure. Control reactions were held at atmospheric pressure for 6 hours. In the absence of any chemical denaturants (i.e., in AmBic buffer alone), digestion at ~80kpsi appears to be the most effective, resulting in almost complete loss of the heavy chain and significant reduction in the light chain, at both temperatures tested. Further increasing pressure to 90kpsi results in a slight reduction in digestion efficiency at both temperatures. **Note that at 55kpsi, a significant benefit of the higher temperature is observed, suggesting that the synergy of temperature and pressure has an impact on reaction kinetics.** Urea has a strong positive effect on digestion at lower pressures (e.g., 45 and 55kpsi), but at higher pressure levels (above ~70kpsi), the combined effect of urea and pressure is detrimental to the trypsin and results in a pressure-dependent reduction in digestion. N-propanol also has a strong positive effect on digestion at low pressures (up to 45kpsi), with the most pronounced benefit at 20kpsi. Like urea, the combination of N-propanol and high pressure (above 45kpsi), leads to loss of trypsin activity and poorer overall digestion. **B.** Unreduced human IgG digested with trypsin at 50°C for 30 minutes, in Ammonium Bicarbonate buffer ± acetonitrile. Reactions were incubated at ambient pressure ("0k"), 45kpsi, or 90kpsi. Trypsin digestion of unreduced IgG is accelerated by incubation at 45kpsi in all three buffers. Reactions carried out in AmBic buffer alone, demonstrate that increasing pressure from 45kpsi to 90kpsi, can significantly accelerate the digestion. However, the combined effect of acetonitrile and 90kpsi pressure, results in a reduction of trypsin activity, leading to poorer digestion at 90kpsi in the presence of the solvent.

Results Part II: Lys-C Digestion at High Pressure

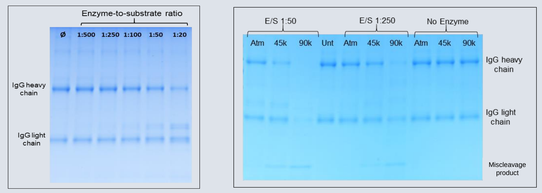


Figure 3. Lys-C digestion of unreduced human IgG is accelerated at high pressure. A. Lys-C digestion of human IgG at atmospheric pressure in non-denaturing buffer requires high enzyme concentration and long reaction time. Unreduced IgG was incubated with Lys-C at the indicated E/S ratio or without enzyme (Ø) for 3.5 hours at 37°C (all at ambient pressure). Some digestion of the IgG heavy chain is observed only at the highest E/S ratio. **B.** Digestion of unreduced IgG at 45kpsi and at 90kpsi is more effective than digestion at atmosphere. Samples were incubated in 100mM AmBic buffer, at 42°C, at the indicated E/S ratio and pressure, for 30min. Untreated control (Unt) is shown for comparison. Little, if any, digestion is observed in the samples incubated at Atm. No non-specific protein loss is observed in the absence of enzyme.

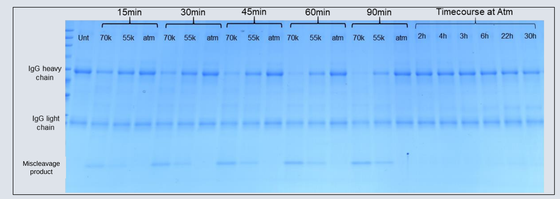


Figure 4. 30 minute digestion of unreduced human IgG at 70kpsi, is more effective than 30 hours at atmospheric pressure. Reactions were incubated at 42°C, at the indicated pressure, for the indicated time with Lys-C (E/S ratio 1:400). Little digestion is observed in the samples at Atm, even after 30 hours. By contrast, at 70kpsi, digestion of the heavy chain is almost complete after 60-90min. Note that digestion of the light chain, and the miscleavage product, can be improved by increasing reaction temperature (see Figure 8, below).

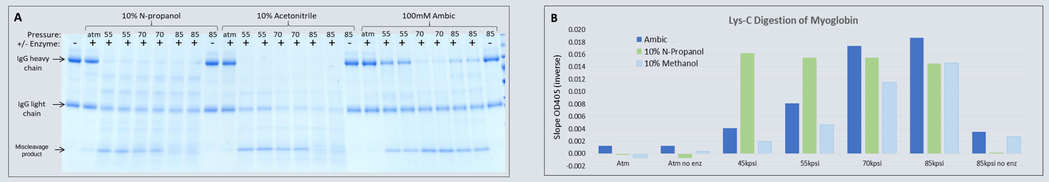


Figure 5. High pressure-accelerated digestion by Lys-C can be further improved by adding organic solvents such as acetonitrile or N-propanol. A. IgG digestions were incubated for 30 min, at 42°C, with Lys-C at 1:400 E/S ratio [controls incubated at 85kpsi without enzyme (-) are also shown], at the indicated pressure (kpsi). Reactions were either in 100mM AmBic buffer alone, or supplemented with 10% acetonitrile or 10% N-propanol. The positive effect of the two organic solvents on high pressure digestion of unreduced IgG is seen by the more complete loss of intact protein on the gel. Little, if any, digestion is observed in the samples incubated at Atm for 30 min, with or without solvents. **Digestion is significantly enhanced at high pressure, and is almost complete in 30 min. at 85kpsi in 10% acetonitrile. B**. **Lys-C activity assay with myoglobin substrate.** All reactions were carried out in 100mM AmBic buffer supplemented with the indicated solvent. Control reactions incubated without enzyme at Atm or at 85kpsi are also shown. Data are presented as inverse slope. As seen for IgG digestion, adding 10% N-propanol improves enzyme activity at pressures up to ~70kpsi. In contrast, the presence of 10% methanol shows no benefit, and may be slightly detrimental to the reaction.

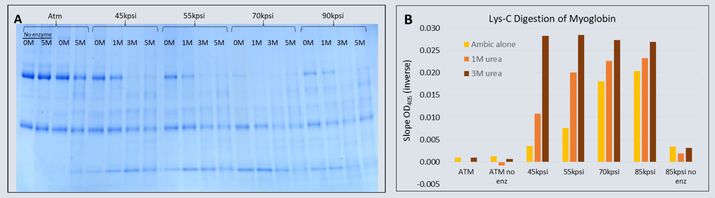


Figure 6. High pressure-accelerated digestion by Lys-C can be performed in the presence of urea. A. Reactions were prepared in 100mM AmBic buffer supplemented with the indicated concentration of urea, and incubated for 30 min at 35°C, at the indicated pressures. Best digestion is observed in 3M urea at all high pressure levels. **Best overall digestion is at 70kpsi in 3M urea. B. Lys-C activity assay with myoglobin substrate**. Reactions were carried out as in panel **A**. Data are presented as inverse slope. In agreement with results in panel **A**, adding 1-3M urea is compatible with, and even improves, Lys-C digestion at pressures up to ~70kpsi.

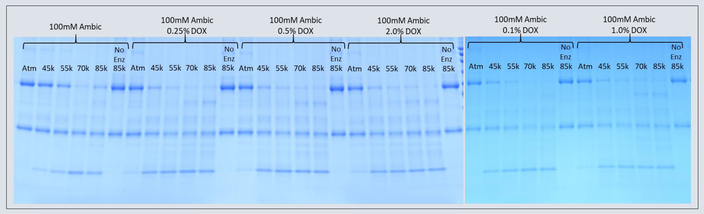


Figure 7. High pressure-accelerated digestion by Lys-C is compatible with Sodium Deoxycholate (DOX). Reactions were incubated at 42°C, for 30 min, in 100mM AmBic buffer with the indicated concentration of Dox, at the indicated pressure (Lys-C 1:400 E/S). Untreated controls incubated at 85kpsi (no enzyme) are shown for comparison. Addition of 0.1% DOX slightly improves digestion above what is seen in AmBic buffer alone. The presence of up to 2% DOX in the reaction is still compatible with digestion at high pressure. **Under these conditions, overall best digestion is at 70kpsi with 0.1% DOX.**

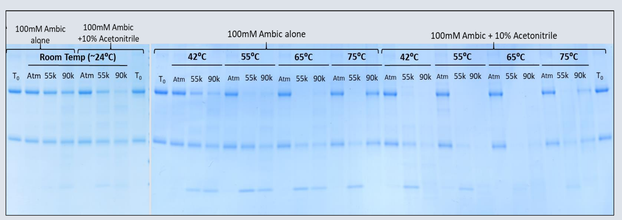


Figure 8. Accelerated Digestion at High Pressure: Synergy of Temperature and Pressure Level. Reactions were incubated for 30 min in 100mM ammonium bicarbonate, with or without 10% acetonitrile, at the indicated temperature and pressure. Untreated controls (T_0) are shown for comparison. **In the moderate temperature range (up to ~55°C), the higher pressure results in more complete digestion and best results are achieved at ~90kpsi.** At higher temperatures (above ~55°C), the combined effect of heat and high pressure on the enzyme structure results in less effective digestion at the highest pressure tested (90kpsi), but better digestion at moderate pressure (55kpsi). **At all temperatures examined, digestion at 55kpsi is significantly more effective than digestion at ambient pressure.** Digestion is further enhanced in the presence of 10% Acetonitrile at all temperatures tested. **Best digestion is observed either at ~90kpsi at 55-65°C, or at 55kpsi at higher temperature.**

Conclusions: Digestion of unreduced IgG by trypsin or Lys-C is shown to be significantly accelerated at high pressure (45-90kpsi) compared to controls at atmospheric pressure (14.7psi). Overall, the best pressure for digestion of native IgG in non-denaturing buffer is ~80kpsi for trypsin , and ~70kpsi for Lys-C. The effect of adding reagents such as urea, acetonitrile, or N-propanol, to the digests was examined, to determine whether the presence of these reagents enhanced, interfered with, or had no effect on, pressure-accelerated digestion.

In ammonium bicarbonate alone, digestion at the higher pressures (70-90kpsi) was more effective than digestion at lower pressures. In the presence of various additives, the combined effect of chemical and pressure-induced denaturation results in improved digestion at moderate pressures (45-55kpsi). Our results with Lys-C suggest that the addition of 10-20% acetonitrile, 10% N-propanol, 1-3M urea, or 0.1% deoxycholate is beneficial to pressure-accelerated digestion. For trypsin digestion, adding 10% N-propanol, 5-10% acetonitrile, or up to 2M urea, can be beneficial to pressure-accelerated digestion at certain pressures.

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