Optimizing Digestion of Hemoglobin Using Chymotrypsin and Assessing Efficiency by Capillary Electrophoresis

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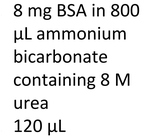
Goals

- Determine an optimal enzyme-to-substrate ratio to digest hemoglobin in its denatured and native form using free and immobilized chymotrypsin enzyme
- Using findings from the separation procedure of the capillary electrophoresis instrument to discover differences of crosslinking reagents—formaldehyde and glutaraldehyde, denatured vs. native hemoglobin, and free vs. immobilized chymotrypsin (CT)

Introduction

- The use of immobilized enzymes to digest protein substrates holds increased experimental advantage in proteomic studies[1].



- Molecular weight: 64 kDa and an oxygen transport metalloprotein[4]
- Iron containing protein possesses four polypeptide chains, each specifically wrapped around a heme group[4]

"The Crystal Structure of Human Deoxyhaemoglobin at 1.74 A Resolution."

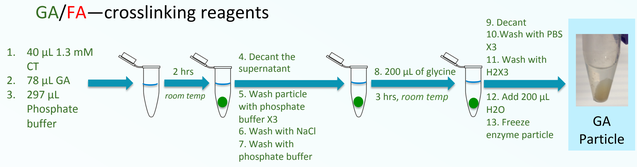
Research conducted investigated the efficiency of hemoglobin digestion in its native and denatured form using free and immobilized chymotrypsin enzyme.

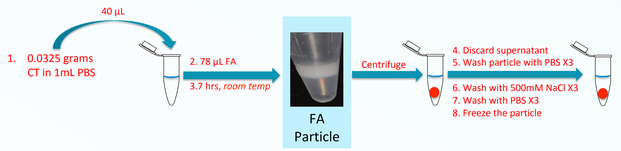
Advantages of Immobilized Enzymes:

1. Increased enzyme-to-substrate ratios[1]
2. Improved protein digestion procedures with little to no autoproteolysis[1]
3. Reusability of the immobilized enzyme particle[1]

Glutaraldehyde (GA) and Formaldehyde (FA)

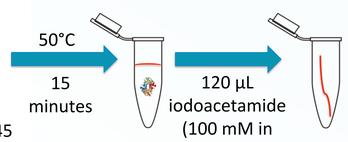
GA/FA—crosslinking reagents





Denaturation of Hemoglobin

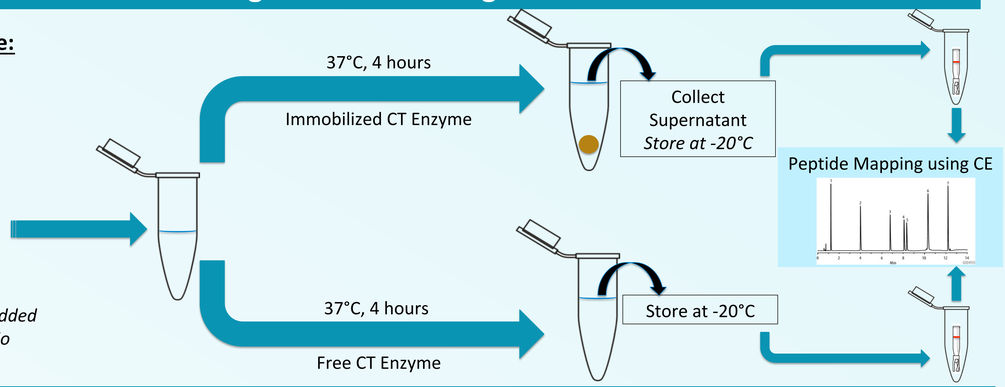
1. 8 mg BSA in 800 µL ammonium bicarbonate containing 8 M urea
2. 120 µL dithiothreitol (45 mM in water)



Denatured Hb.

Digestion of Hemoglobin

Digestion Procedure:



Denatured vs. Native Hemoglobin

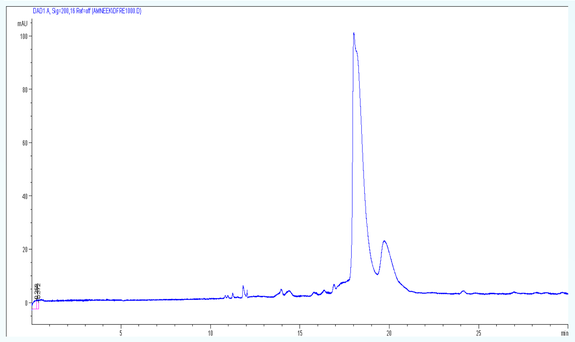


Figure 1 Denatured Hemoglobin proteolysis
Denatured hemoglobin (0.65 mM) digested with Free CT (0.0065 mM) at 37°C for 4 hours

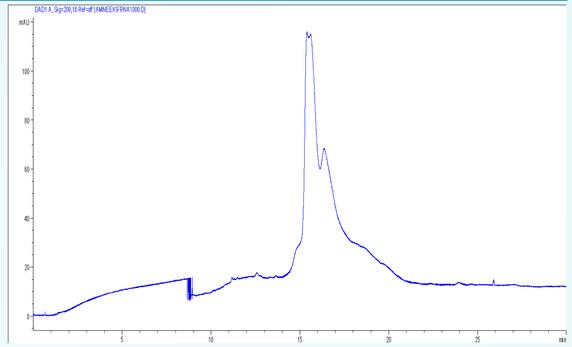


Figure 2 Native Hemoglobin proteolysis
Native hemoglobin (0.65 mM) digested with Free CT (0.0065 mM) at 37°C for 4 hours

Glutaraldehyde vs. Formaldehyde

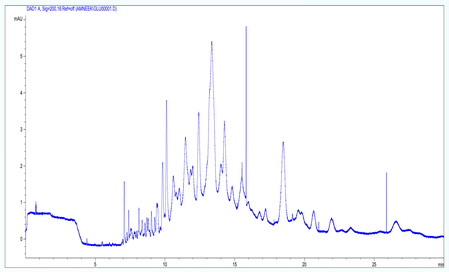


Figure 3 Denatured Hb. (1.3 mg) digested using Immobilized CT/ Glutaraldehyde (1.3mg) at 37°C for 4 hours

Extensively used for the immobilization of enzymes, glutaraldehyde serves as a reliable crosslinking reagent.
Figure 1.3 displays a 1:1 enzyme to substrate digestion.

Chymotrypsin immobilized with crosslinking reagents:
Glutaraldehyde
&
Formaldehyde
used to digest denatured hemoglobin

Glutaraldehyde displayed greater digestion efficiency. This is supported by the presence of smaller peaks on the electropherogram for denatured hemoglobin digested using immobilized CT/Glutaraldehyde

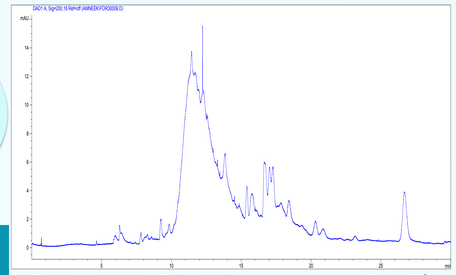


Figure 4 Denatured Hb. (1.3 mg) digested using Immobilized CT/ Formaldehyde (1.3 mg) at 37°C for 4 hours

Formaldehyde is used to *trap* the CT enzyme to support proteomic analysis and detection of interactions b/w substrate and enzyme[3].
Figure 1.4 displays a 1:1 enzyme to substrate digestion.

Enzyme to Substrate Ratio 1:1 vs. 100:1

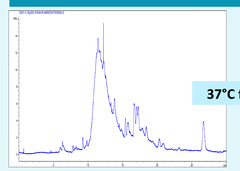


Figure 5 Denatured Hb (1.3 mg) digested with GA/CT (1.3 mg)

37°C for 4 hours

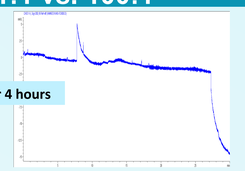


Figure 6 Denatured Hb (0.1733 mM) digested with GA/CT (0.001733 mM)

- 1:1: Better digestion—smaller peaks and no *noise*
- 100:1: no defined peak + *noise*

Free vs. Immobilized Enzyme

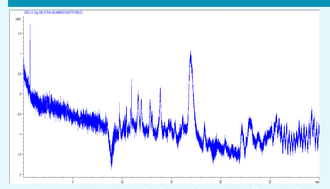


Figure 7 Denatured Hb (0.325 mM) digested with Free CT (0.0325 mM)

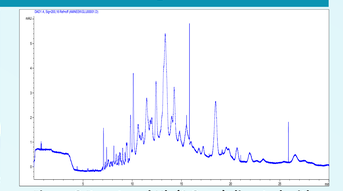


Figure 8 Denatured Hb (1.3 mg) digested with Immobilized CT/GA (1.3 mg)

The free CT electropherogram displays increased *noise,* with uncertainties as peaks are not definitive. Immobilized CT electropherogram has greater # of peaks which are more defined w/o *noise*

Conclusion

- Use of denatured hemoglobin allows for a more accessible substrate, resulting in a greater digestion efficiency
- GA/CT 1:1 (E:S) ratio showed enhanced digestion efficiency compared to 100:1
- Crosslinking reagent glutaraldehyde portrayed better results for a digestion procedure compared to formaldehyde
- Versatile and possessing greater effectiveness immobilized enzyme proved ideal digestion for proteomic studies over the use of free enzyme

Future Work

Immobilized agents and enzymes will be used to fabricate IMERs (immobilized enzyme microreactors). The IMER will later be coupled to a capillary electrophoresis instrument, allowing for the analysis of large biomolecules through on-line digestion of proteins.

- Optimization of the IMER:
  - Increase/Decrease pressure
  - Increasing time of the digestion—increasing interaction between immobilized enzyme and denatured hemoglobin
  - Increase time of fabrication—immobilized enzyme interacts with the capillary for a longer period of time

References

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2. Perutz, et al. "The Crystal Structure of Human Deoxyhaemoglobin at 1.74 A Resolution." *J.Mol.Biol.*, www.rcsb.org/pdb/explore/jmol.do?structureId=4hhb&bionumber=1&jmolMode=HTML5.
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4. "Metalloprotein." *Metalloprotein - an Overview | ScienceDirect Topics*, www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/metalloprotein.