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Nucleophilic labeling as a tool to identify protease substrates

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Nucleophilic Labeling as a Tool to Identify Protease Substrates

by

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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of the

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For my mother Morrine, my father Linwood,

and my brothers, Micaiah and Adiel

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I would like to thank God for all blessings

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INTRODUCTION

Proteases are an important class of enzymes. They are involved in a number of biological processes. Some notable proteases studied in the Craik Lab include MTSP-1 which may play a role in tumor growth (Bhatt,2005) and KSHV protease which plays a role in the life cycle of the virus (Deckman,1992). Key to understanding a protease's biological function is the identification of its natural substrates.

There are different methods for identifying a protease's natural substrates. One method utilized in the Craik Lab is a computational / bioinformatics approach. First positional scanning synthetic combinatorial libraries (PS-SCL) developed in the Craik Lab (Harris,2000) identifies the preferred amino acid sequence N-terminal to the cleaved peptide bond (non-prime). Residues C-terminal to the cleaved peptide bond (prime) can be identified with substrate phage display (Harris, 1998). The optimal amino acid sequence is then used to scan the genome to look for possible matches. This approach yields many candidate substrates and is necessarily coupled to another approach to narrow the field. Another method screens *in vitro* small-pool cDNA expression libraries for substrates by addition of recombinant protease. While these methods work, they are laborious, time-consuming and generally only allow a substrate to be found on an individual basis.

The goal of the project is to develop a "chemical screen" using nucleophilic capture of acylated substrates. This method would allow substrates to be found in a more rapid and comprehensive manner. The new method of nucleophilic labeling is analogous

to, and in fact exploits, the normal catalytic machinery. Figure 1 shows a schematic of the labeling mechanism.

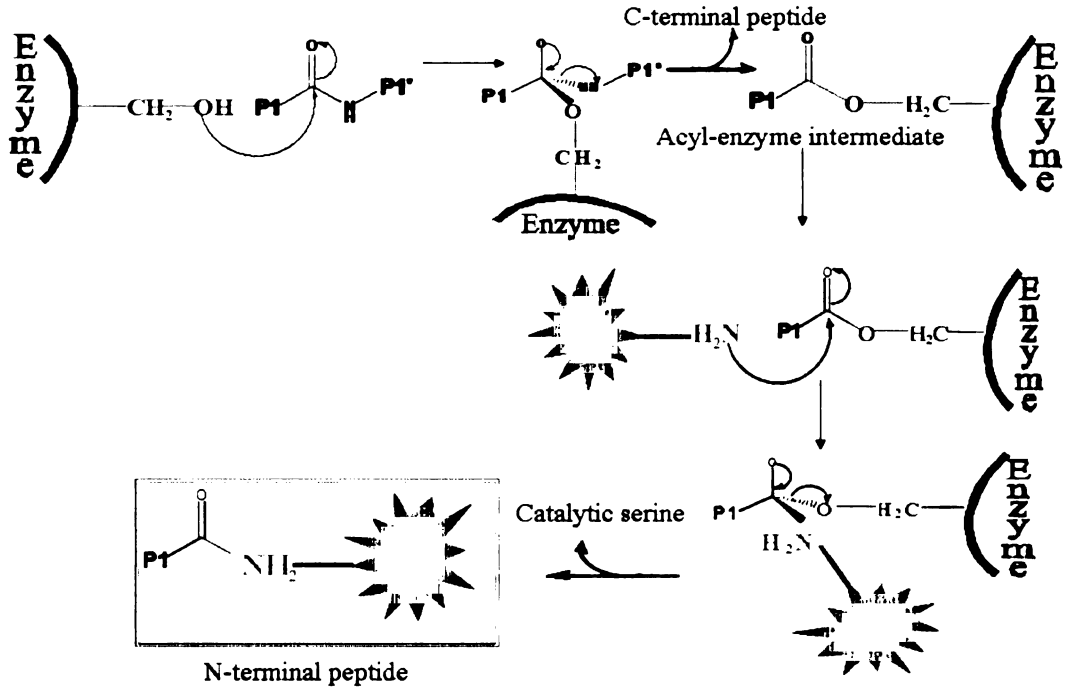

Figure 1. Mechanism of Nucleophilic Labeling

During catalysis serine and cysteine proteases bind their substrates in the pocket of the enzyme. The catalytic serine attacks the carbonyl of the scissile bond (cleaved peptide bond) forming a tetrahedral intermediate. The collapse of the intermediate results in the release of the C-terminal fragment of the substrates and formation of an acyl-enzyme intermediate with the N-terminal portion of the substrate covalently attached to the enzyme. It is important to note that the prime side pocket is now open. Next, water deacylates the acyl-enzyme intermediate regenerating the protease and releasing the N-terminal fragment. Using the chemical screen strategy an alternate nucleophile competes

with water to deacylate the acyl-enzyme intermediate. Upon deacylation, the alternate nucleophile labels or tags the N-terminal fragment.

Substrate labels that deacylate a specific protease require three basic elements. First is the nucleophilic warhead which competes with hydrolysis. Second is the specificity element corresponding to the optimal prime side amino acid sequence. Though substrate recognition is primarily found on the non-prime side there is also some specificity on the prime side to varying degrees. Previous studies have shown that peptides with prime side specificity deacylate substrates more efficiently (Wang,1996). The third element is a reporter tag that allows labeled substrates to be monitored and/or isolated.

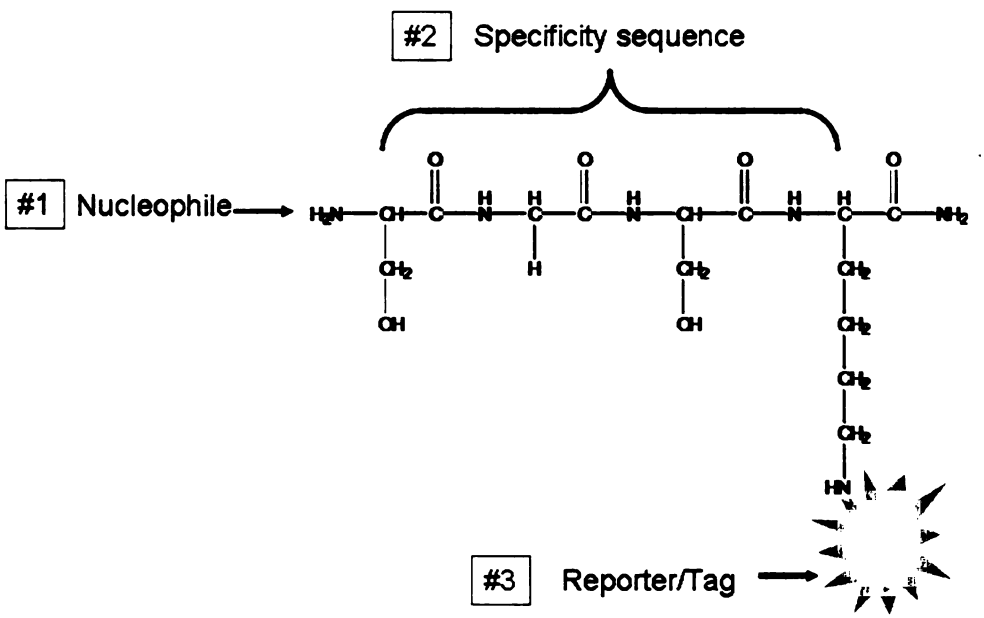

Figure 2. Primary elements of nucleophilic label

The previous study has shown that a dipeptide can be incorporated into the N-terminal fragment of BSA using neutrophil elastase, and cathepsin S. The labels used

required millimolar concentrations and specificity was not tested. The goal of this thesis is to explore the aspects of the nucleophilic label to improve the efficacy of the system.

In order to further develop nucleophilic labeling as a tool to identify proteases substrates, a model system is chosen using Granzyme B (GrB) for initial study. Granzyme B is a serine protease of the trypsin-fold with a standard His-Asp-Ser catalytic triad. It is one of several granzymes that have been identified in humans known to be involved in the apoptotic pathway (Hudig,1987), a necessary part of the immune system designed to rid the body of virus- and tumor- infected cells. Granzyme B is unique because it is the only known mammalian serine protease with a preference for cleaving after aspartate residues.

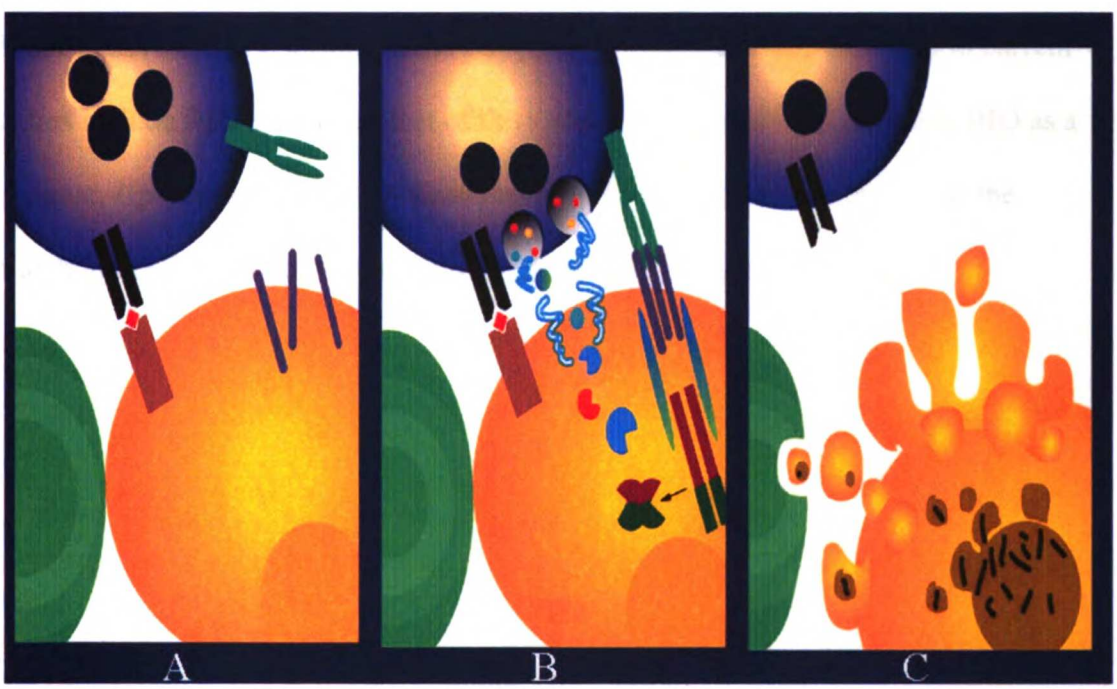

Figure 3. Cytotoxic lymphocyte (CTL) mediated cell death. **(A)** Effector cells recognize target cells. **(B)** Next the exocytosis of granules containing proteases delivers a lethal hit, aided by perforin to the target cell. **(C)** Finally apoptosis ensues through by series of intracellular proteolytic events.

GrB is an ideal model protease to develop the chemical screen for several reasons. GrB can be expressed recombinantly in yeast. The crystal structure (Waugh,2000) has been solved thus allowing protein engineering studies. Analysis using PS-SCL has determined the preferred non-prime side amino acid sequence for granzyme B to be IEPD. Using substrate-phage display, the primes side specificity of Granzyme B has been determined (Harris, 1998). Glycine is preferred at the P2' position while no charged residues are allowed at P1. Finally, previous studies have already validated several substrates and implicated others. Though little else is known about the requirements at the other prime sites, analysis of some of the known substrates can provide insight into amino acids accepted, if not preferred, at the other sites. For example, caspase substrates (3 and 7) have a serine located at the P1' position. Several substrates used in current studies include BID and HIP. Most of the research discussed involves using BID as a substrate. Upon activation, BID stimulates the release of cytochrome C from the mitochondria thereby conveying a proapoptotic signal. (Luo,1998).

MATERIALS AND METHODS

Synthesis of MCA and Biotinylated Peptides

FMOC amino acids are obtained from Novabiochem. BOC-HNOGly-OH used for the synthesis of the alkoxyamine is a generous gift from the Ellman lab (Berkeley). Rink AM Amide resin (Novabiochem #01-64-0038) is preswollen by shaking in N,N dimethylformamide (DMF) for at least 30 minutes. Next the resin is deprotected with 20% piperidine in DMF for 1 hour. Three molar excess of amino acids are dissolved in DMF. Three molar excess of coupling reagents PyBOP (Novabiochem# 01-62-0016), HOBt (Sigma H-2006), and DIPEA are added and the reaction is allowed to proceed for 1 hour. After 1 hour the resin is rinsed thoroughly with DMF. Next 20% piperidine in DMF is used to deprotect the n-terminus. The process in repeated until synthesis of target sequence is complete. The resin in then washed 3X with DMF, 2X methanol, and 2X with acetone (acetone wash is not performed when synthesizing alkoxyamine labels). The resin is then allowed to dry under vacuum. After drying the peptide is cleaved from the resin with 2 ml 95% TFA, 2.5% water, and 2.5% triisoproprysilane (TIS) shaking gently for 1 hour. After cleavage the peptide is collected in a glass tube. The resin is washed once with 1 ml of 100% TFA. The wash is also collected. The collected peptide solution is then added dropwise to 45 ml of diethyl ether and allowed to precipitate overnight at -80°C. The precipitate is spun down and dried. The peptide is then dissolved in appropriate solvent to purify by HPLC.

Synthesis of FAM Peptides

To synthesize FAM labeled peptides, after the final coupling, the peptide is cleaved with TFA and precipitated with ether as previously described. The N-terminus is left FMOC protected. The dried peptide is added to a final concentration of 0.1mM –0.2mM in DMF. The label, 5-(and –6) carboxyfluorescein succinimydl ester (Molecular Probes# C1311) is added at a ratio of 1:2 (FAM:peptide). DIPEA is added at 1.5 equivalents to peptide. The reaction proceeds overnight while shaking and protected from light. The N-terminus is then deprotected by adding piperidine to a final concentration of 20% and then proceeds for 1 hour. Again the peptide is ether precipitated.

Peptide Purification and Characterization

All peptides where purified on reversed-phase C18 column (Vydac# 218TP1022) or Xterra reversed-phase C18 (Waters# 186001930). The organic phase solvent is 95% acetonitrile with 0.0825% TFA in water. Aqueous phase consists of 0.1% TFA in water. Purity of peptides is accessed on the Agilent 1100 system using a reverse-phased C18 analytical column (Vydac# 281TP104). Peptides are characterized by MALDI-TOF using the Voyager Biospectrometry Workstation in linear mode.

Labeling denatured BSA with MCA peptide label

BSA at a concentration of 100 μM with 50 nM granzyme B in 0.1 M borate buffer, pH = 8 is used for the cleavage assay. To prepare the denatured BSA sample, 100 μL of a 200 μM stock BSA was boiled for 5 minutes. The reaction is incubated in a 37°C water bath. Time points are taken at 1.5, 3, 6 and 21.5 hours. The samples are run on a 4-20% tris-glycine gel (Novex from Invitrogen) then stained with coomassie to visualize.

For the labeling assay with the methoxycourmarin label stocks are prepared by lyophilizing appropriate volume of label for 0.05, 0.1, 0.2, 0.4, 0.8, and 1.6 mM label. After being dried, each aliquot is dissolved in borate buffer. Heat denatured BSA and GrB are added to a final concentration of 20 μM BSA and 75 nM GrB respectively. Samples are placed at 37°C. Time points are taken at 60 and 210 minutes. The MultiImage Light Cabinet (Alpha Innotech Corp) is used visualize labeled protein.

Labeling denatured BSA with FAM peptide label

Samples are prepared using 10 μM heat denatured BSA and 50 nM GrB. A volume of 5 μL of label dissolved in DMSO is added to final concentrations of 2.15 mM, 1.1 mM, and 540 μM. A buffer containing 50 mM HEPES, 100 mM NaCl, pH = 8 is used instead of borate buffer. Again reactions are allowed to proceed at 37°C with time points taken at 15 minutes, 2 hours, and 12 hours. Coomassie staining is used to follow proteolysis while the Typhoon fluorescence imager is used to visualize labeled protein.

Effects of detergent in labeling denatured BSA with FAM peptide labels

To determine the significance of detergent, samples are prepared using 10 μM dBSA, 50 nM GrB, and 2 mM FAM label in HEPES buffer with and without Tween 20 detergent. Samples are placed at 37°C for 1 hour. Again coomassie staining and Typhoon are used for visualization.

Labeling the Natural Substrate, BID with a Biotinylated Peptide

Human recombinant BID at a concentration of 1.5 μM of (R&D Biosystems cat# 2006), with 0.75, 1.5, 3, 6, and 12 mM label are added to HEPES buffer with no detergent. After addition of granzyme B to a final concentration of 100 nM, sample is incubated at 37°C for 1 hour. The reaction is quenched by adding TFA to a final concentration of 0.4%. The general protocol to remove un-reacted label is described below.

Microcon Processing to Eliminate Excess Label

Two buffers are used during microcon processing: (1) processing buffer which consists of 50 mM MES pH=6.0, 100 mM NaCl, 0.4% TFA and (2) ecotin processing buffer which combines 1 μL of 434 μM IEPD-ecotin stock / 50 μL of GrB storage buffer (50 mM MES pH=6.0, 100 mM NaCl).

50 μL of sample and 450 μL of quench buffer are added to a Microcon YM-3 (Millipore; Cat #42404). The sample is spun 14,000xg for 60 min. After the spin 500 μL

of quench buffer to microcon to retentate. After transferring microcon unit to new tube the sample is spun again at 14,000xg for 60 min. This process is repeated twice for a total of 3, 60 minute spins. After a fourth spin at 75 min, 100 μL of Ecotin quench is added to retentate and the sample is spun at 14,000xg for between 35 min to 40 min. Then 10 μL 2x Tricine sample buffer with TCEP is added and retentate is retrieved by inverting Microcon and spinning for 1,000xg for 3 min. The membrane can be washed with additional 2x Tricine sample buffer and retrieve as described above if necessary.

Quenching Methods Experiment

Four identical samples are prepared with 1.5 μM BID, 100 nM GrB, and 6 mM label (E+S+L). A proteolysis control sample (E+S) is prepared without label. To prepare inhibited GrB stock (E*I), 1 μL of 10 mM a small molecule inhibitor (Thornberry group #L-008969, Merck) is added to 2.9 μM GrB and allowed to preincubate for 15 min. A sample is prepared with 1.5 μM BID and 100 nM E*I. A sample using 1.5 μM BID, inhibited GrB stock, and 6 mM label is also prepared. All samples are prepared in HEPES buffer. Reactions are placed at 37°C for 1 hour. After 1 hour, one E+S+L sample is quenched by adding TFA to a final concentration of 0.4%, one sample is quenched using the Merck inhibitor 15 minutes before processing in microcon, another is quenched with 2 μL of 434 μM ecotin for 15 minutes. The fourth sample receives no quenching treatment. Samples prepared with inhibited enzyme stock also receive no quenching treatment. The proteolysis control sample is treated with TFA. An additional sample prepared with 1.5 μM BID and inhibited enzyme and allowed to incubate for 15 minutes.

The sample is then immediately processed in a microcon. Samples E+S+L quenched with TFA and the proteolysis control are processed as normal. All other samples are processed with omission of the ecotin quench step.

Effects of pH on Labeling BID

To determine the effect of pH the following buffers are used: 50 mM MES, 100 mM NaCl, pH = 6 (GrB storage buffer); 50 mM PIPES, 100 mM NaCl, pH = 7; and 50 mM HEPES, 100 mM NaCl, pH = 8 (GrB activity buffer). The reaction consists of 1.5 μM BID, 100 nM GrB, and 6 mM label. After 1 hour, the reactions are quenched with 400 μL of 250 μM of the Merck inhibitor in the appropriate pH buffer. The sample are then processed as previously described with the exception that 2 μL of 434 μM IEPD-ecotin was added to all samples (except the pH 8 to 4) for the second spin.

Protein Engineering to Improve Substrate Specificity

All mutagenesis reactions followed the Quikchange protocol (Stratagene) and other standard procedures. The K41A/Y151A mutant was constructed by first introducing the K41A mutant into the wild-type rat granzyme B gene in pPICZαA (Invitrogen). After verification of the K41A mutant, the Y151A was then engineered into the K41A template, generating K41A/Y151A.

Labeling reactions are prepared using 1.5 μM BID, and approximately 100 nM of K41A/Y151A granzyme B mutant in HEPES buffer defined previously. Label concentration is varied.

Increasing the Reactivity of the Nucleophile

Reactions samples are prepared by mixing 1.5 μM BID and 2 μM HIP proteins and 11.3 μM to 2.9 mM label in HEPE buffer. Samples are placed at 37°C for 1 hour then quenched with TFA and acetone. Quench samples are prepared by added TFA and acetone to BID/HIP cocktail, then adding label and incubating at 37°C for 1 hour.

MALDI-TOF Analysis of BID labeling Reactions

To produce samples to analyze by MALDI, two 21 μL samples containing 81 μM BID, 100 nM GrB, and 6 mM label in detergent free GrB activity buffer are prepared. The samples proceed for overnight at 37°C. The two samples are combined and processed in microcons pre-blocked with BSA. An on-plate desalting procedure, described below is performed before analysis. Both α-cyano-4 hydroxy cinnamic acid and sinapinic are used as matrices.

On-Plate Desalting Method

1. Combine and vortex 1 μL of analyte and 1 μL of matrix.
2. Spot 1 μL of sample and allow spot to dry.
3. Wash spot
    a. After spot has dried, add 3 μL of 0.1% TFA in water (or 0.1% FA).
    b. Wait 30 seconds to 1 minute then aspirate.
4. Allow spot to dry.

RESULTS

Synthesis and Purification of Nucleophilic Peptide Labels

The optimal prime side sequence for GrB is XG where X is uncharged. Caspase 3 and 7, validated granzyme B substrates, have the prime side sequence of SG. Serine is placed in the P3' position to give the peptide label flexibility. A lysine in P4' position allows the reporter tag to be placed on the side chain and should be far enough away from the prime side binding pocket to prevent steric hindrance. Lysine with the fluorophore, methoxycoumarin (MCA) attached at the side chain is commercially available. The peptide sequence S-G-S-K(MCA) is chosen for the first peptide label. Figure 4-1 to 4-8 shows the structures of labels described in the study.

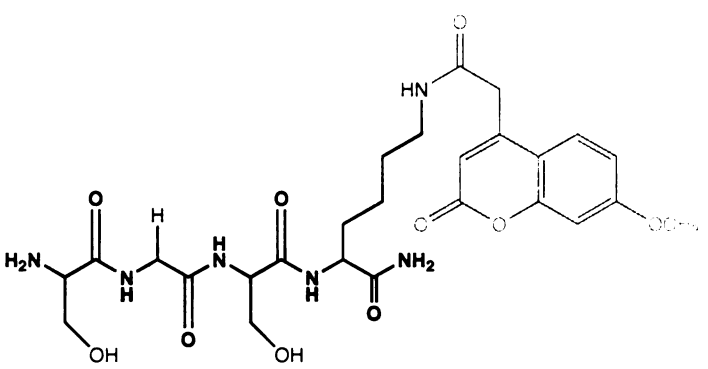

Fig. 4-1. H_2N-SGSK(MCA)

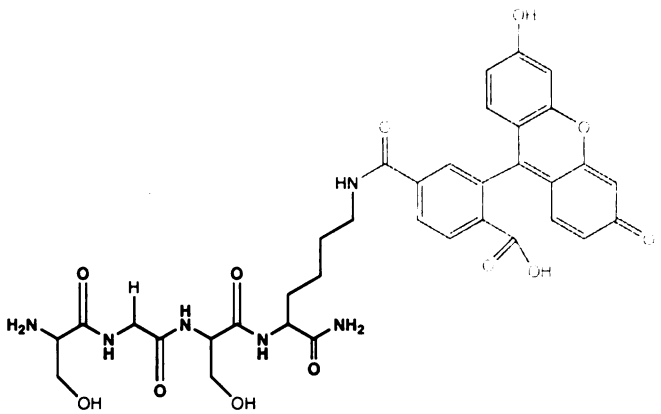

Fig. 4-2. H_2N-SGSK(FAM)

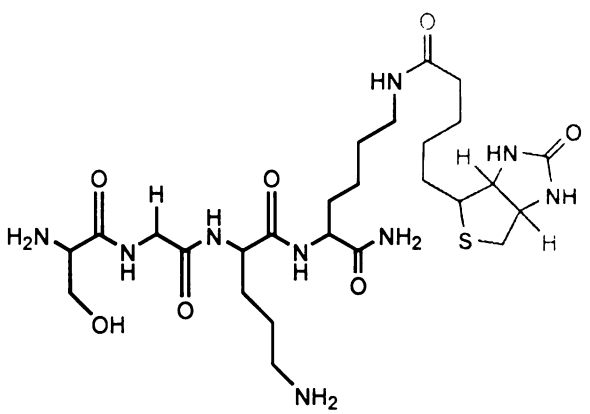

Fig. 4-3. H_2N-SGSK(Biotin)

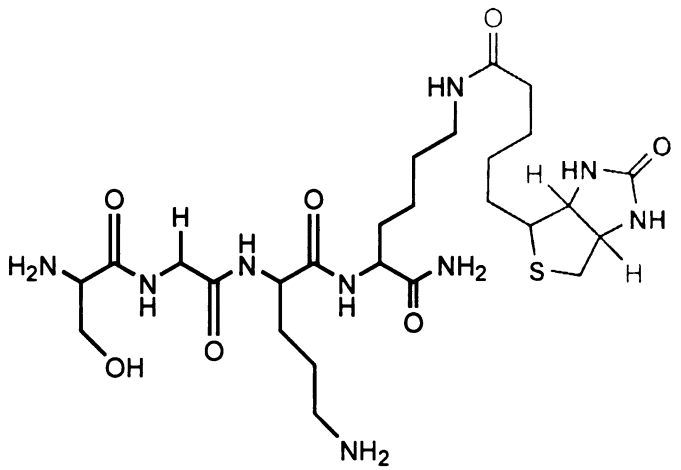

Fig. 4-4. Knob label #1:H_2N-SGOrnK(Biotin)

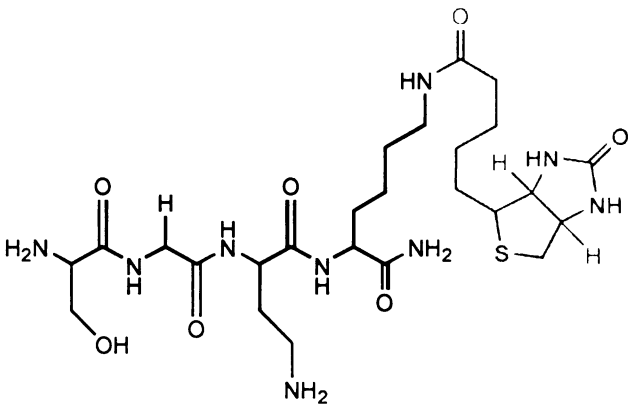

Fig. 4-5. Knob label #2:H_2N-SGDabK(Biotin)

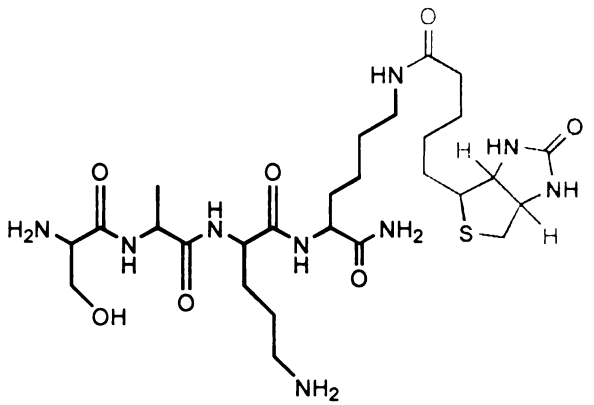

Fig. 4-6. Knob label #3:H_2N-SAOrnK(Biotin)

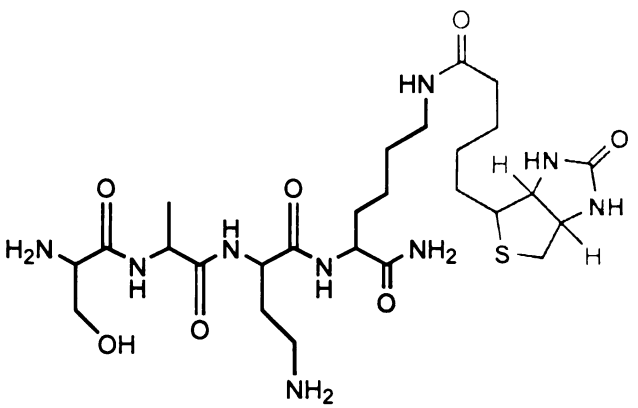

Fig. 4-7. Knob label # 4:H_2N-SADabK(Biotin)

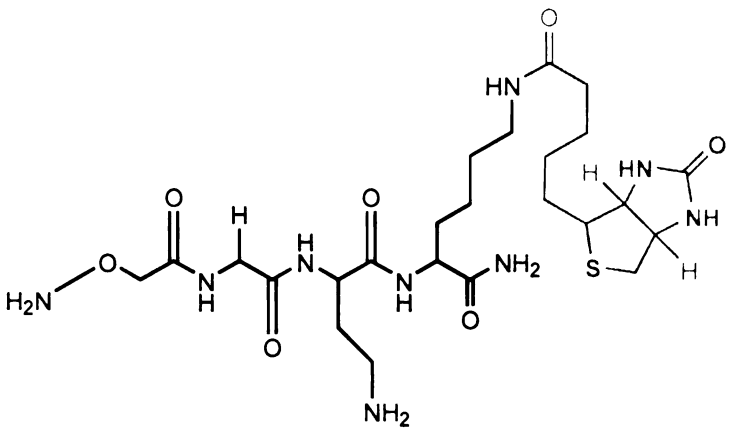

Fig. 4-8. Alkoxyamine label:H_2N-GADabK(Biotin)

Purification and analysis of "Wild-type" label (wt. label) and knob label #4 are shown as examples.

Figure 5-1 shows the preparative RP-HPLC trace. Wt. peptide is purified until eluted as a single peak at 9.068 min by preparative RP-HPLC.

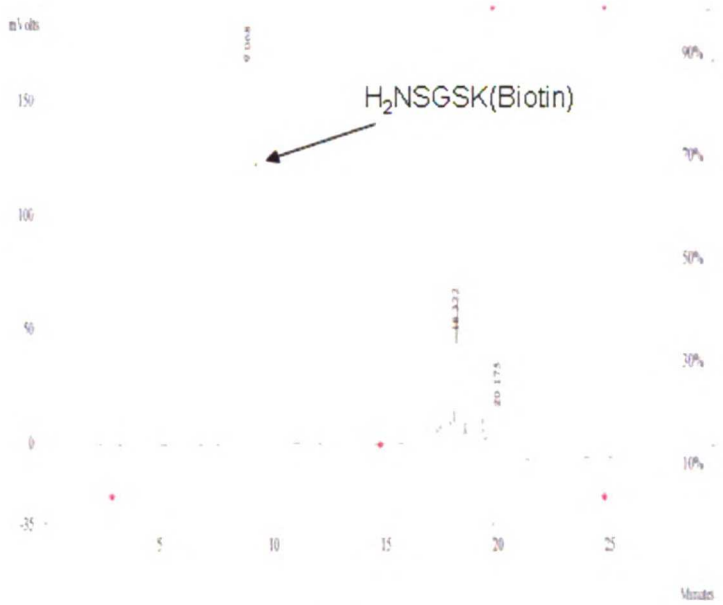

Figure 5-1. Purification of wt. Peptide label

MALDI-TOF verifies the correct mass of the peptide (figure 8-2 shown below).

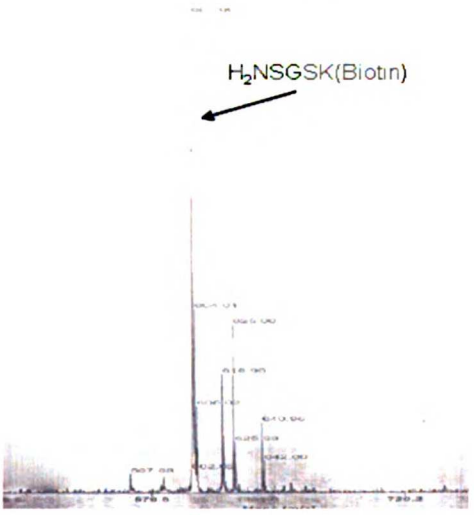

Figure 5-2. MALDI of wt. peptide label

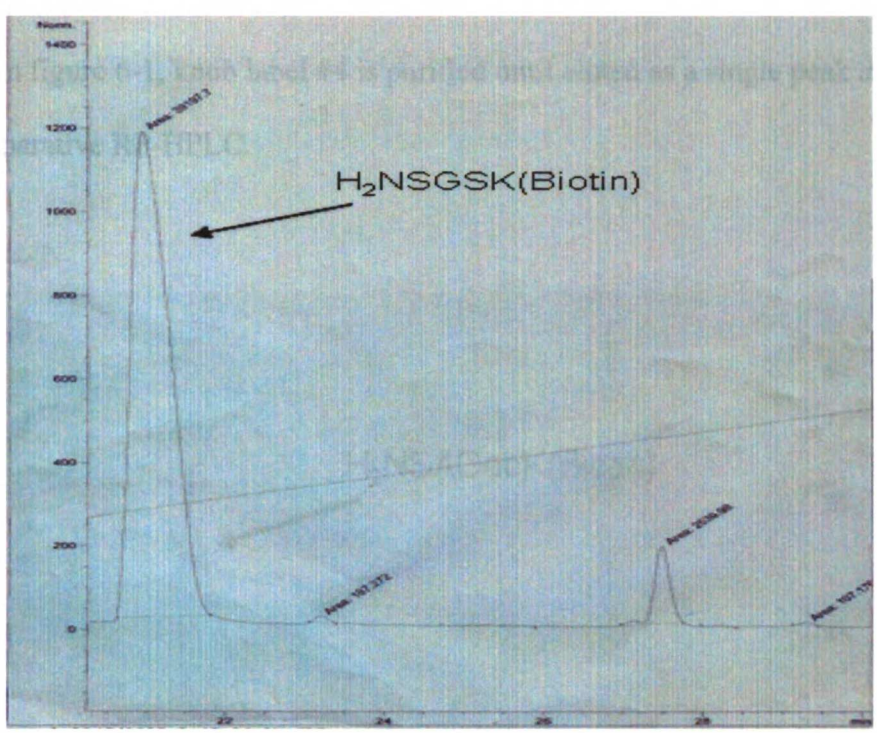

Fig. 5-3. Analytical HPLC of wt. label

Analytical RP-HPLC reveals the purity to be 93% (figure 5-3 shown above).

As shown in figure 6-1, knob label #4 is purified until eluted as a single peak at 9.123 min by preparative RP-HPLC.

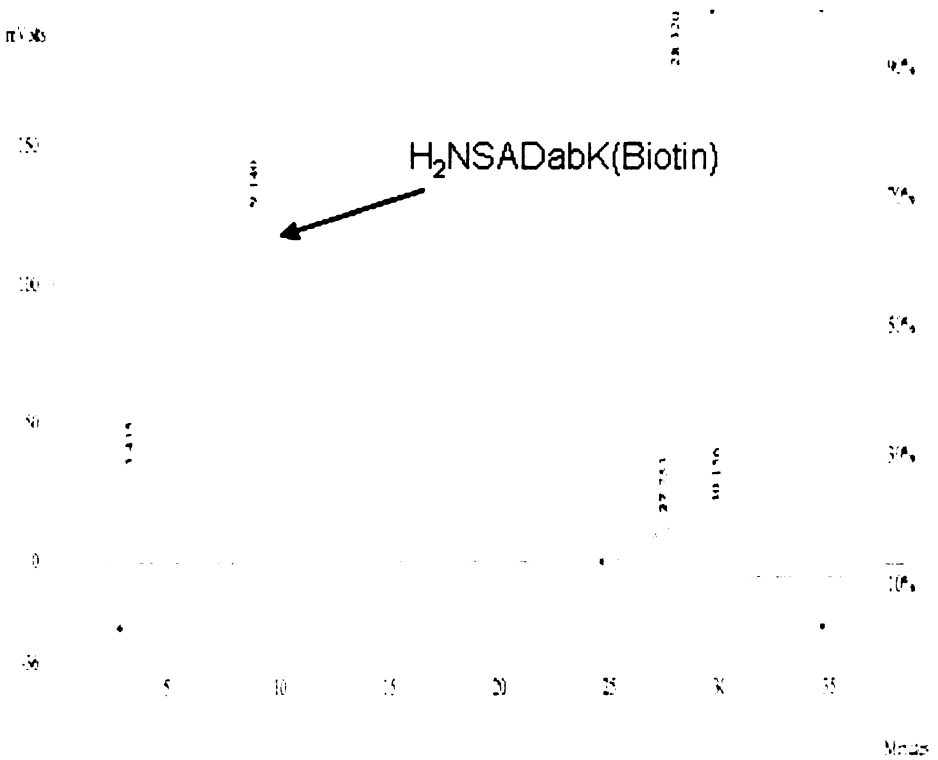

Fig. 6-1. Purification of knob label #4

MALDI-TOF verifies the correct mass of the peptide (figure 6-2 shown below)

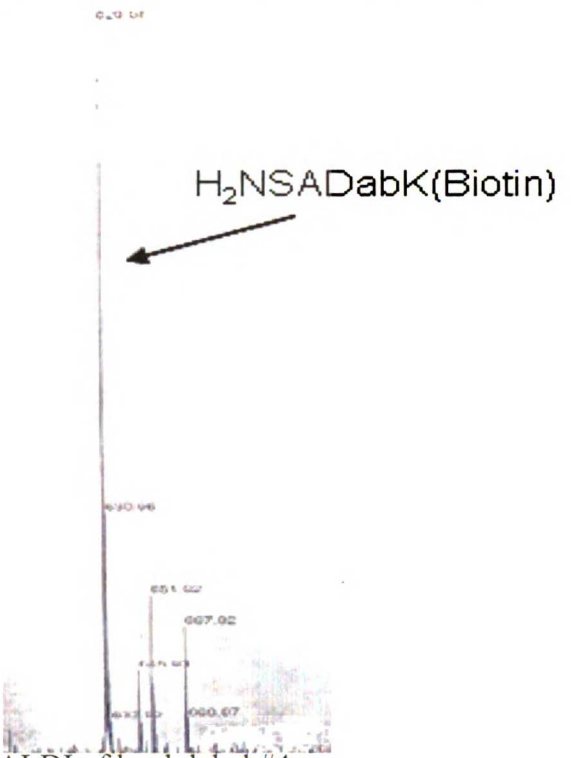

Fig. 6-2 MALDI of knob label #4

Analytical RP-HPLC reveals the purity to be 94% (figure 6-3 shown below)

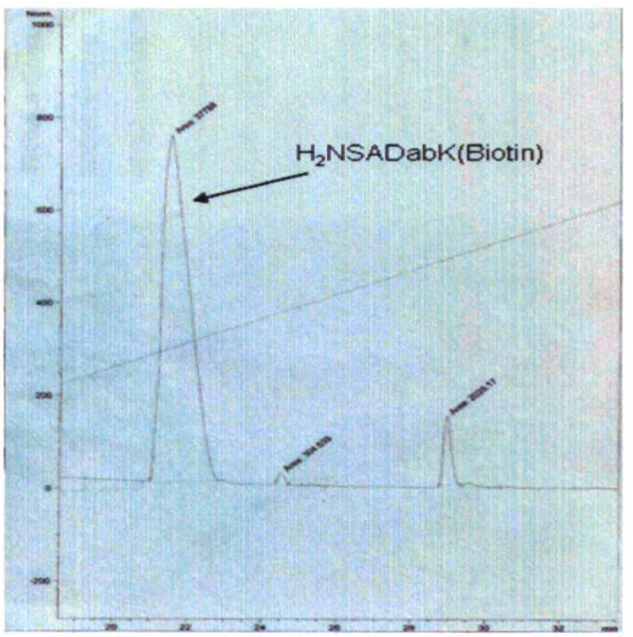

Fig. 6-3. Analytical HPLC of knob label #4

Establishing a Baseline

Labeling denatured BSA with MCA Peptide Label

To develop a model system, several substrates are investigated for their ability to be proteolyzed by granzyme B. Bovine Serum Albumin (BSA) is chosen as an abundant and inexpensive option. A sequence search revealed two possible cleavage sites in, LVTD-LT and VEKD-AI. A schematic is shown in figure 7. The sequence VEKD-AI is predicted to be the better granzyme B site based on PS-SCL and phage display.

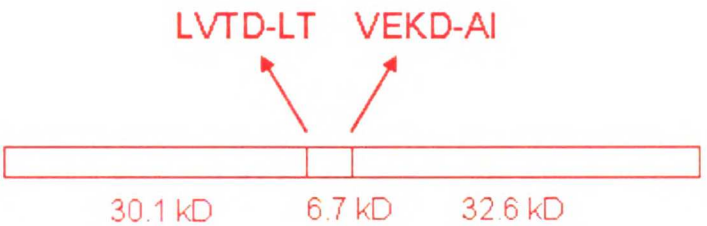

Figure 7. Schematic of GrB cleavage site of BSA

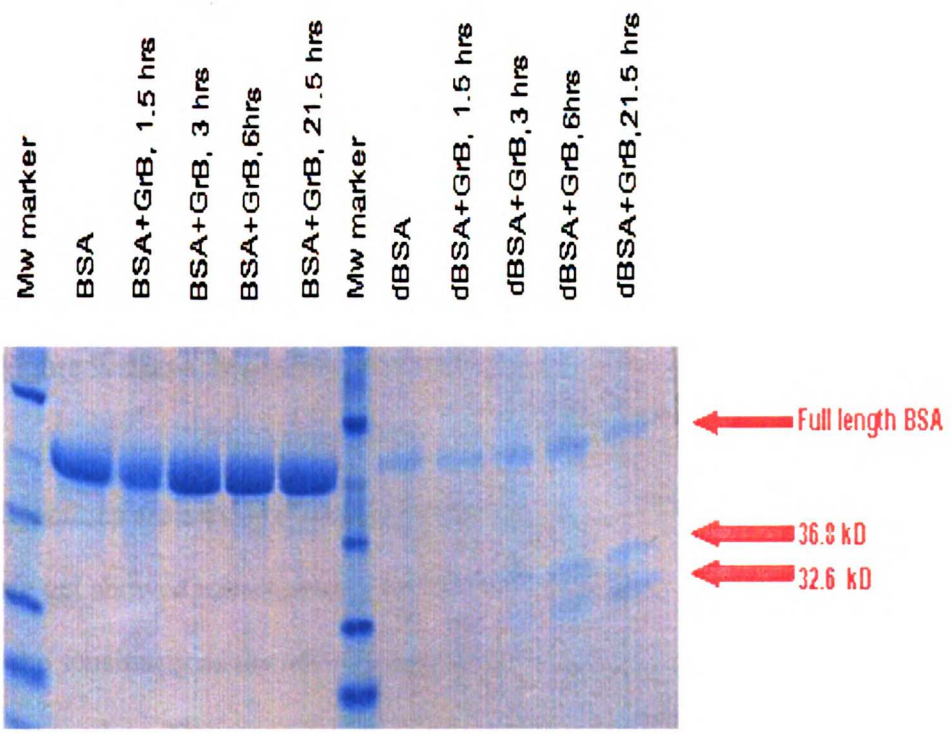

Figure 8. Cleavage of dBSA by GrB

Figure 8 shows a coomassie stained gel of proteolyzed BSA. Lanes 3 through 6 show BSA incubated in the presence of GrB at 37°C. Lanes 9 through 12 shows BSA that has been heated denatured. As seen in the coomassie, only the heated denatured BSA (dBSA) is proteolyzed by GrB. Significant proteolysis in evident after 6 hours. dBSA is found to be cut by granzyme B resulting in a 32.6 kDa C-terminal fragment and a 36.8 N-terminal fragment.

After establishing that dBSA can be cleaved by GrB, proteolysis is conducted in the presence of label. Figure 9 shows all possible fragments generated from cleavage at VEKDAI and/or LVTDLT sites. The star denotes possible labeled N-terminal products. Based on previous results the 36.8 kDa fragment is expected be labeled.

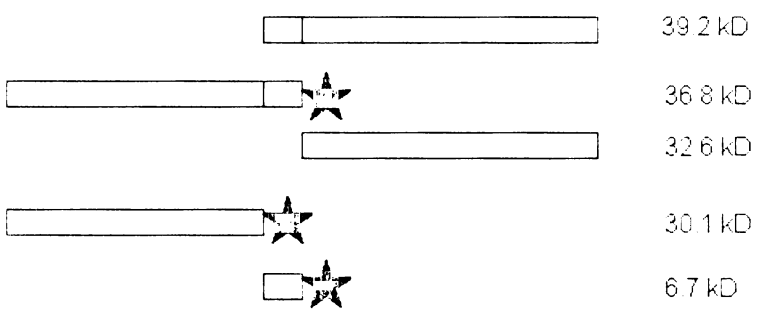

Figure 9. dBSA fragments produced by proteolysis by GrB

Reactions are carried out as a function of label concentration and time. The coomassie gel showed robust proteolysis after 6 hours. Earlier time points were taken because the sensitivity of the MCA label should be able to detect lower levels of labeled N-terminal product. The scans in figures 13 and 14 show spots at 0.8 and 1.6 mM around the molecular weight of the 36.8 kDa N-terminal fragment. The higher molecular weight

band may correspond to full-length BSA. This is not unexpected considering the "sticky" nature of dBSA. The lowest spot reveals what could be labeled product due to secondary cleavage. Cleavage at both possible granzyme B sites would produce a very low molecular weight fragment of 6.7 kDa (see figure 9). Controls from label alone sample (data not shown) does not show the lower spot.

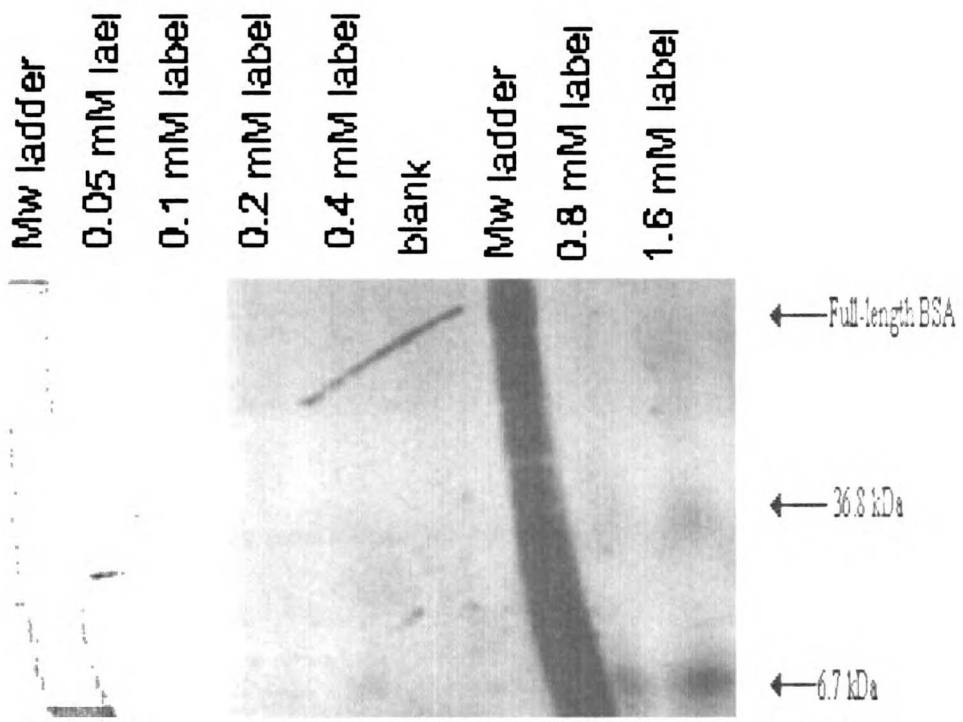

Figure 10. Fluorescence Scan of BSA with MCA peptide at 60 minutes

Figure 11. Fluorescence scan of Labeling of BSA with MCA peptide at 210 minutes

Labeling dBSA with the methoxycoumarin label produces inconclusive blobs. The current gel imager uses a filter with an excitation wavelength of 595 ± 25nm. This is too high to provide sufficient sensitivity and resolution using MCA. In addition label instability from photobleaching leads to reduced sensitivity. FAM is a fluorophore that can be used instead of MCA. FAM is more stable and its spectral wavelengths are compatible with the Typhoon imager.

Labeling denatured BSA with FAM peptide label

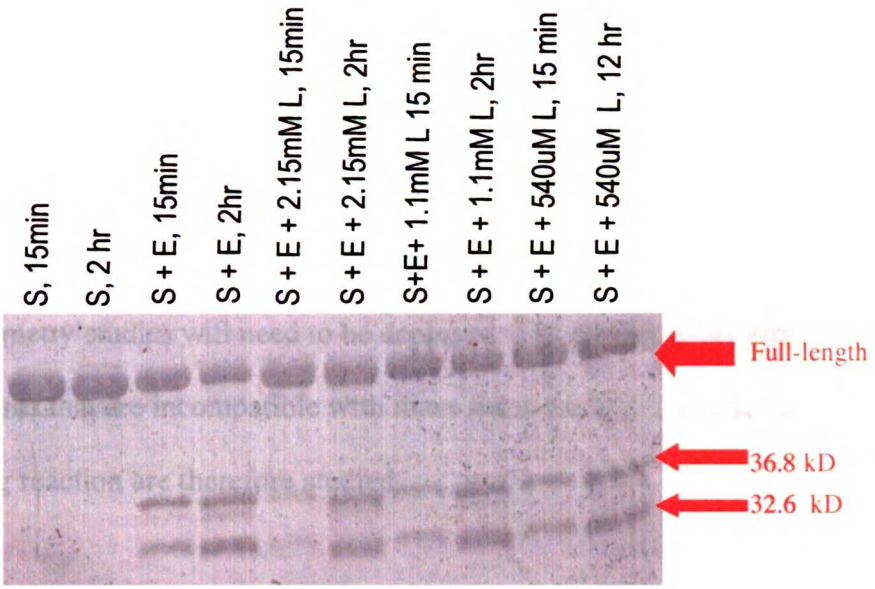

Fig. 12-1 Coomassie gel of BSA labeling with FAM

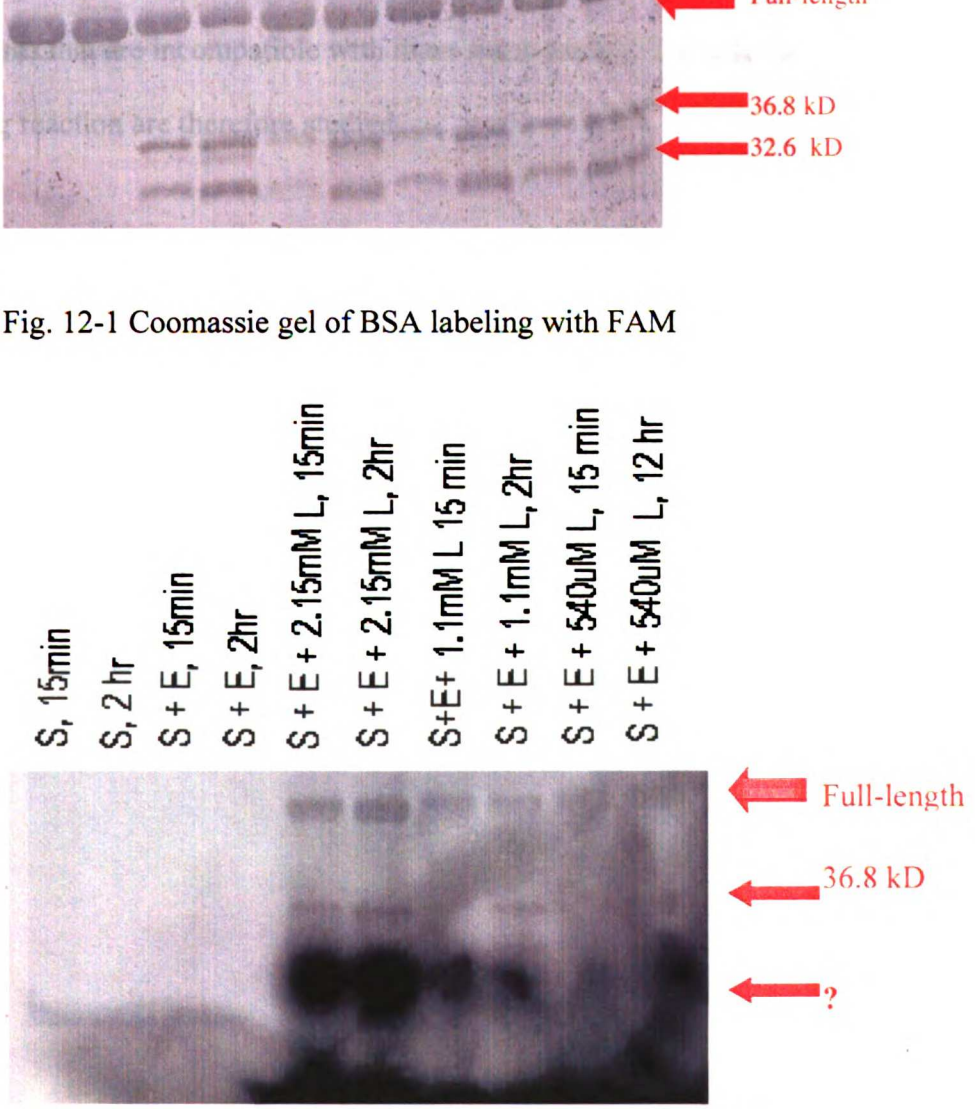

Figure 12-2. Fluorescence scan of BSA labeling with FAM

Bands are now discernible in the fluorescence scan shown in figure 12-2. As seen with the methoxycoumarin labels, the fluorescence scan reveals evidence of nonspecific labeling of full length BSA. The lower band corresponds to what could be labeled N-terminal product. The "spot" previously thought to be labeled product is still evident.

Visualization is improved with the use of the FAM peptide label. There now appears to be evidence of labeled product. In order to gain better insight, mass spectrometry studies will need to be deployed. The current assay employs the use of detergents that are incompatible with mass spectrometry. The effects of detergent on the labeling reaction are therefore studied.

Effects of detergent in labeling denatured BSA with FAM peptide labels

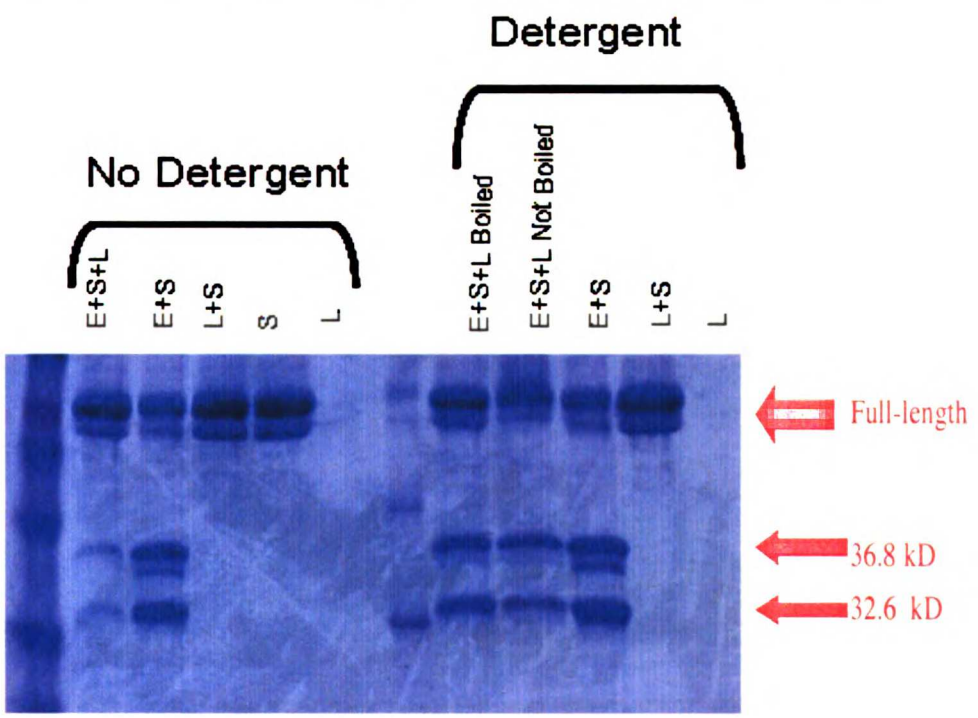

Fig. 13-1. Coomassie gel of BSA detergent analysis

The coomassie-stained gel shown in figure 13-1 reveals the importance of detergent. Lane 2 shows proteolysis of dBSA in buffer containing no detergent with label present. Proteolysis is markedly reduced compared to samples in lanes 8 and 9 which have detergent. However, when looking at the enzyme plus substrate lanes 3 and 10, there is little difference in the degree of proteolysis whether detergent is present or not. This suggests that in the absence of detergent, the label has an inhibitory affect on proteolysis.

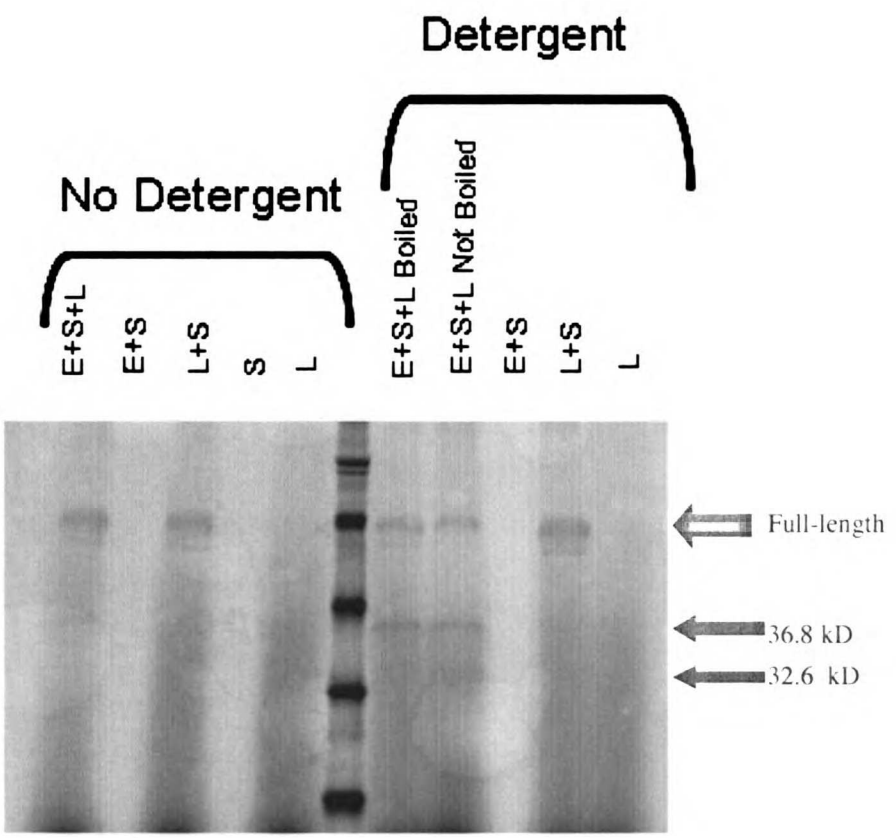

Fig. 13-2. Fluorescence scan of BSA detergent analysis

Once again, the fluorescence scan reveals nonspecific labeling of full-length BSA. Presumed labeled product is only evident when detergent is used as in lanes #7 and 8

Labeling the Natural Substrate, BID with a Biotinylated Peptide

Complications persisted throughout the previous studies. Denatured BSA is not an optimal substrate for GrB. In order to optimize proteolysis without the use of detergent, a natural substrate provides the best hope. BID is a validated GrB substrate that is available recombinantly. In order to eliminate continued issues with visualization, biotin will replace the use of a fluorophore.

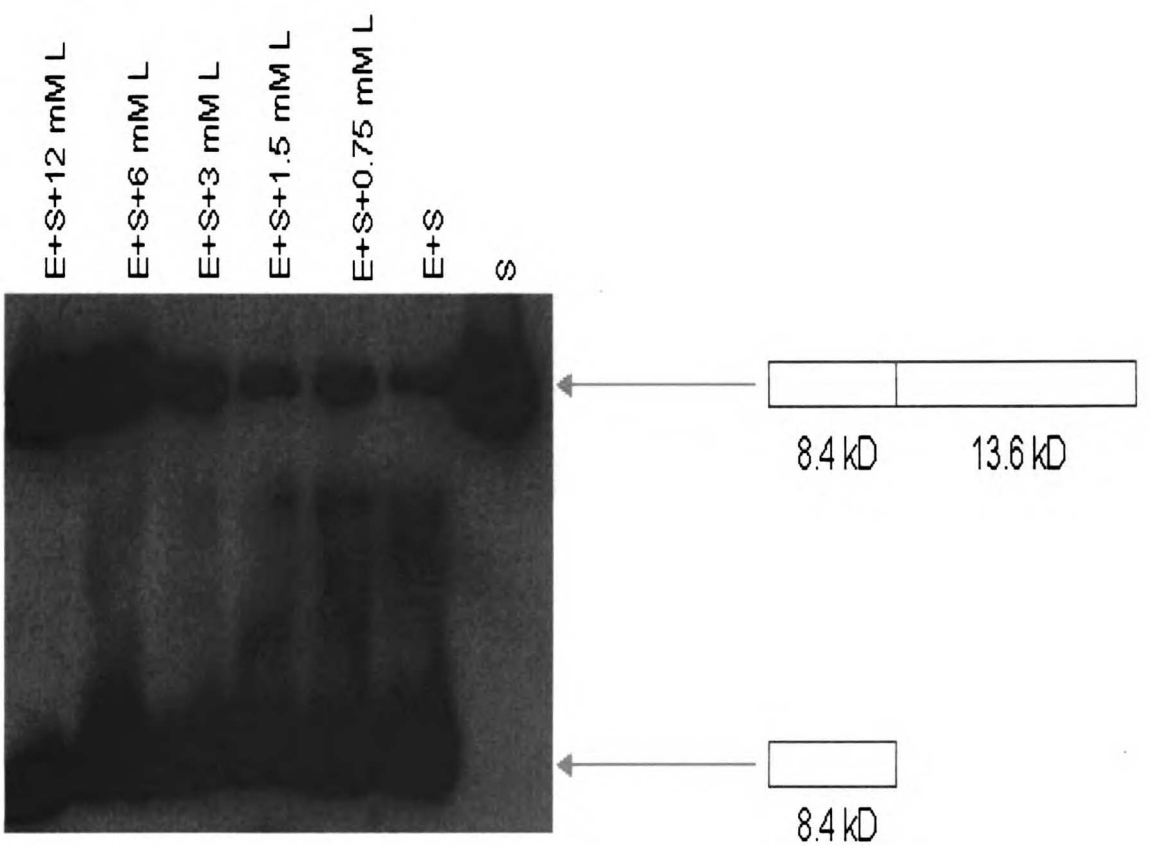

Figure 14-1. Anti-BID blot detects N-terminal fragment of proteolyzed BID

Proteolysis of BID by granzyme B produces two fragments; a 13.6 kDa C-terminal fragment and an 8.4 kDa N-terminal fragment. Only the N-terminal fragment is detectable by the BID antibody. The BID blot (figure 14-1) shows proteolysis decreases as label concentration increases from 0.75 mM in lane # 5 to 12 mM in lane, with marked reduction in proteolysis above 3 mM thus suggesting that the label has some inhibitory affect when compared to the proteolysis control lane #6.

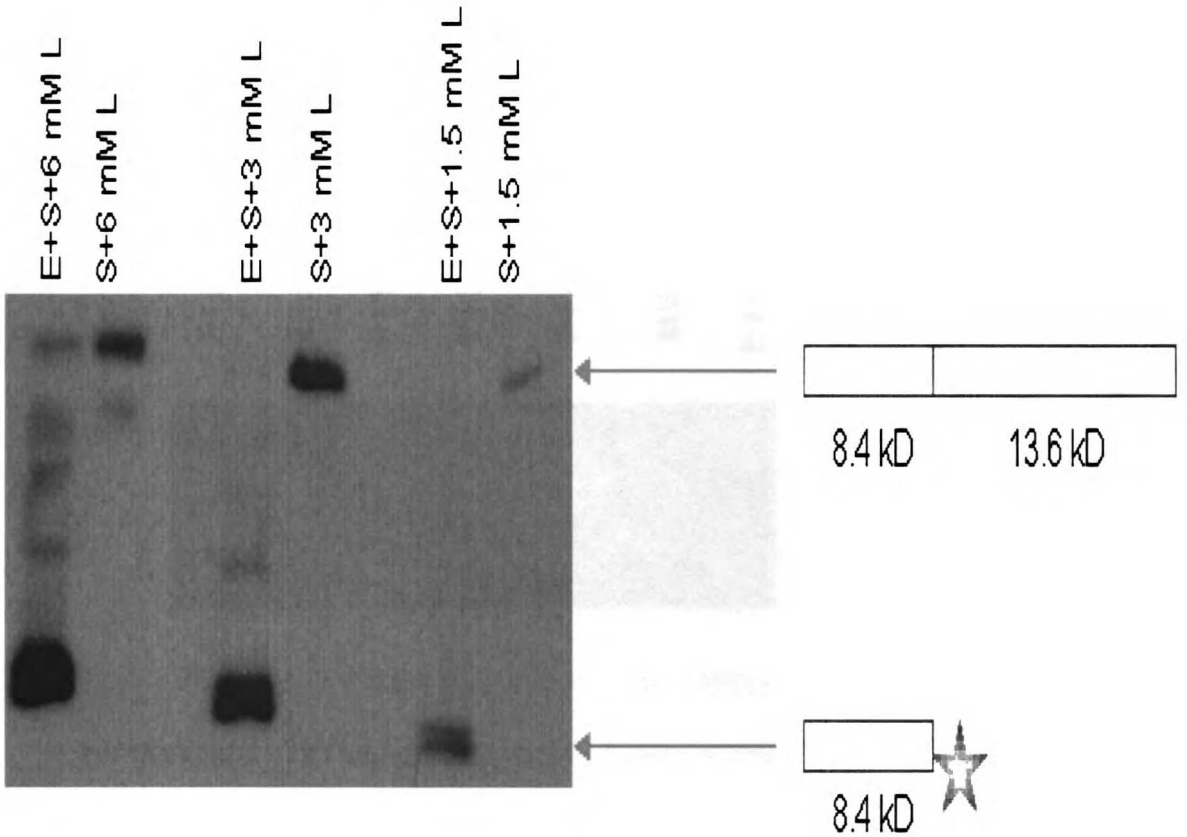

Figure 14-2. Avidin blot shows labeling of N-terminal fragment of BID

Label concentrations of 1.5, 3, and 6 mM are chosen to examine labeling of the N-terminal fragment of BID. Again, the blot shows some background labeling of full-length BID. Labeling of N-terminal product is clearly evident. Results of the avidin blot

against the biotinylated peptide label indicate that the N-terminal 8.4 kDa fragment of BID is labeled in a concentration dependent manner. There is significantly more N-terminally labeled BID at 6 mM versus 1.5 mM.

To determine the conditions at which the labeling of the N-terminal fragment is observed, various quenching methods are explored.

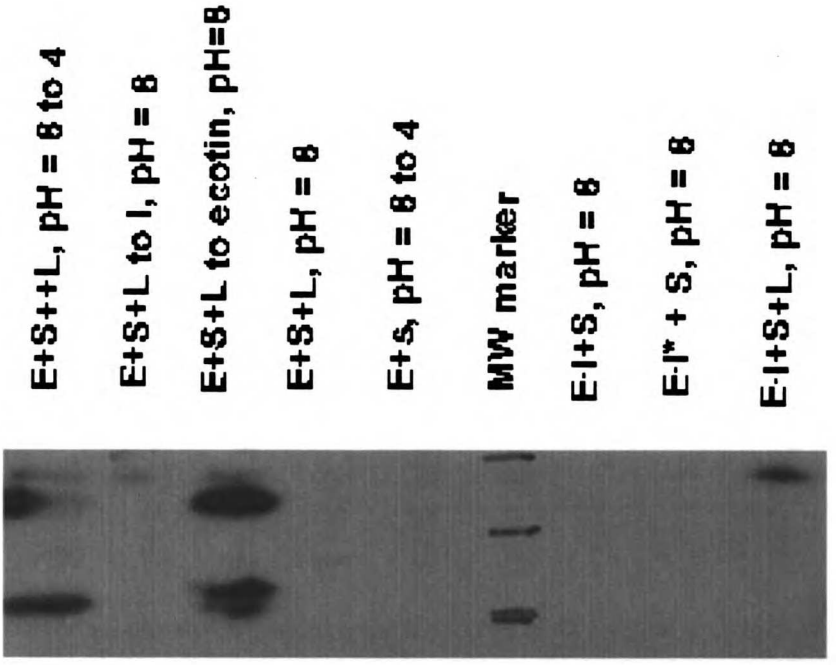

Figure 15-1. Avidin blot of Quenching Methods

For the avidin blot (figure 15-1) lane #1 shows "normal" labeling conditions in which the granzyme B and presumably the peptide label is deactivated, "quenched" upon addition of 4% TFA. Once again the blot reveals prominent labeling of the N-terminal fragment of BID at 8.4 kDa with slight labeling of full-length BID. Lane #2 shows a reaction in which granzyme B is quenched by a small molecule inhibitor at pH 8. The blot reveals no evidence of labeled product or full-length protein. Similarly, lane #3 uses a macromolecular inhibitor, ecotin to quench the labeling reaction. However, unlike the

small molecule inhibitor the sample shows labeling of N-terminal product. The blot also shows significant labeling of full-length BID even compared to a "normal" reaction. Lane #4 shows a reaction kept at an active pH of 8. Like lane #2, there is no evidence of labeling of N-terminal product or full-length BID. For the sample shown in lane #9, inhibited enzyme is added to substrate in the presence of label at an active pH 8. As expected, the blot shows slight labeling of full-length BID the same as substrate plus label controls.

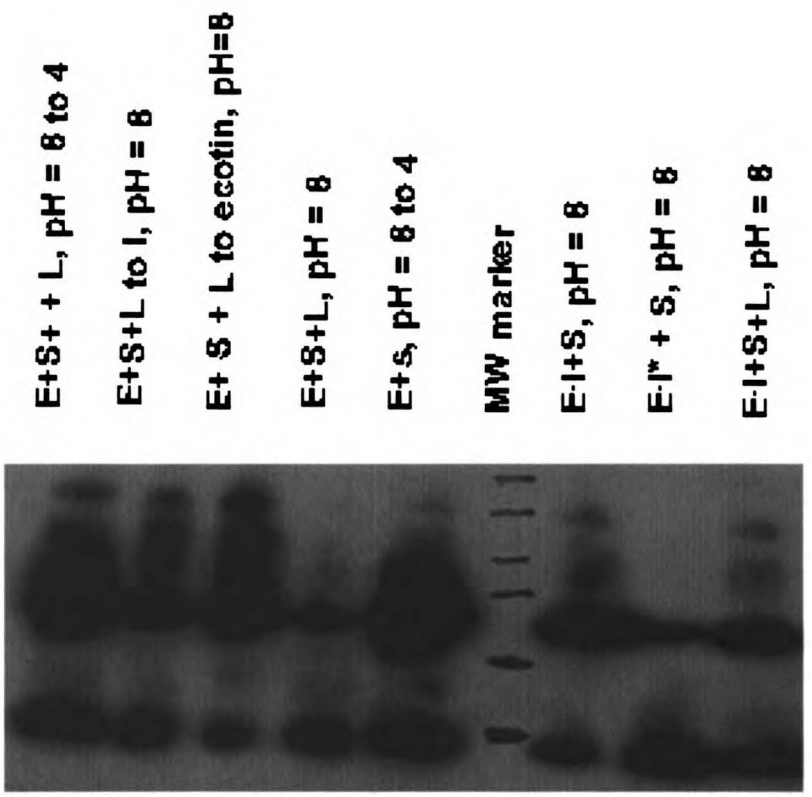

Figure 15-2. Anti-BID blot of Quenching Methods

The BID blot shown in figure 15-2 verifies that proteolysis occurred for all reactions in which active enzyme in used regardless of whether or not nucleophilic

labeling has occurred. Lanes #7, 8, and 9 indicate proteolysis even though inhibited enzyme is used suggesting an effective, acid-free quench still needs to be developed.

It is expected that pH plays a role in the labeling protein substrates. Previous results indicate that observed labeling of N-terminal peptide occurs when the proteolysis occurs at pH 8 and then proteolysis is quenched with ecotin as well as TFA which drops the pH to approximately 3.5 In order to access the sensitivity of the labeling reaction to pH, the labeling reaction was performed at pH 6, 7, and 8.

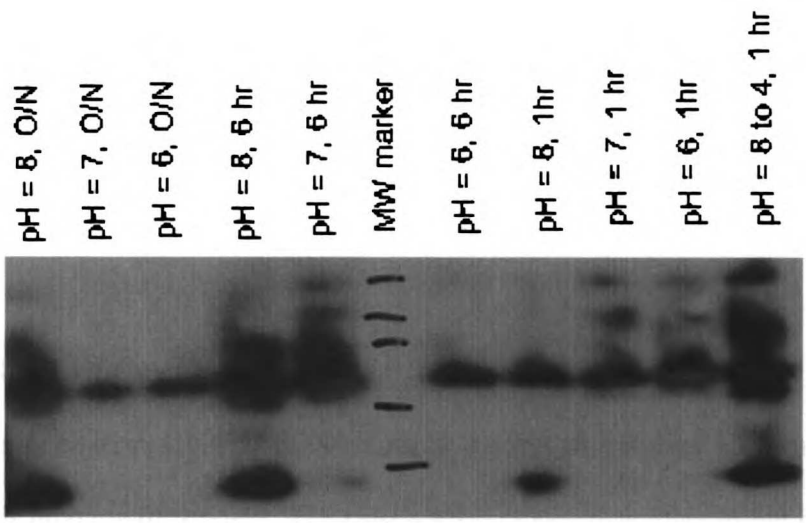

Figure 16-1. Anti-BID blot of pH studies

The BID blot indicates that significant proteolysis only occurs at pH 8 (lanes #1, 4, 8, and 11). Not surprisingly on the avidin blot (figure 16-2), N-terminal labeling is only seen for these time points. Samples in lanes #4, 8, and 11which are quenched with inhibitor show notably reduced labeling when compared to normal reactions conditions using TFA as a quench as seen in lane #1. This is explained by previous experiments showing that the inhibitor does not quench GrB for the duration of the processing.

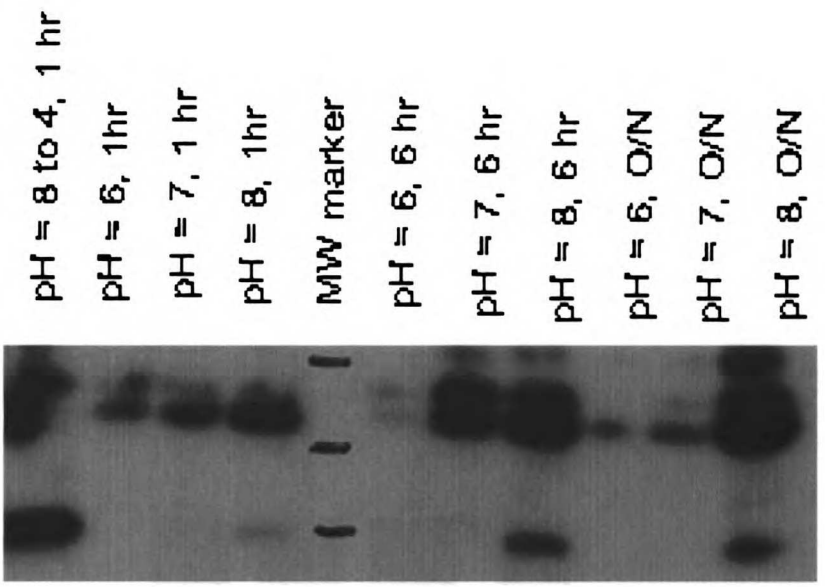

Figure 16-2. Avidin blot of pH studies

Improving the Labeling System

We now observe labeling of N-terminal proteolytic product. Controls suggest that the labeling is enzyme dependent. The next course of action is to improve labeling by increasing the specificity of the label and to increase potency of the nucleophile. Increased labeled product will provide the best chance for characterization by mass spectrometry and allow lower concentrations of label to be used.

Protein Engineering to Improve Substrate Specificity

Most of the substrate recognition and binding of a substrate for granzyme B takes place on the non-prime side (Waugh,2000). This leads to concerns over the ability of the peptide label to recognize granzyme B versus other serine and cysteine proteases in a cell

extract. In order to increase specificity, a unique interaction is developed. A mutant granzyme B, K41A/Y151A is engineered. This modification introduces a new cavity. To complement this cavity, a "knob" label with a side chain to fit into the cavity is made. Several knob labels are explored. Granzyme B K41A/Y151A is efficient to cleavage BID (data not shown).

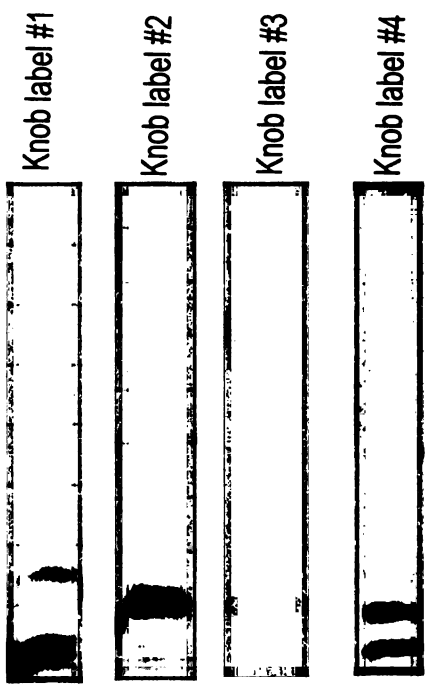

Figure 17. Knob labels with GrB mutant

Avidin blot (figure 17 above) results of using the engineered protease show for knob labels #1, 2, and 4 two N-terminal labeled fragments. This suggests that secondary cleavage which is confirmed by LC-MS/MS.

Increasing the Reactivity of the Nucleophile

The alternate nucleophile has to compete with water, at 55 M, to deacylate the substrate. One way to up the odds in favor of the label is to increase the reactivity of the nucleophilic label.

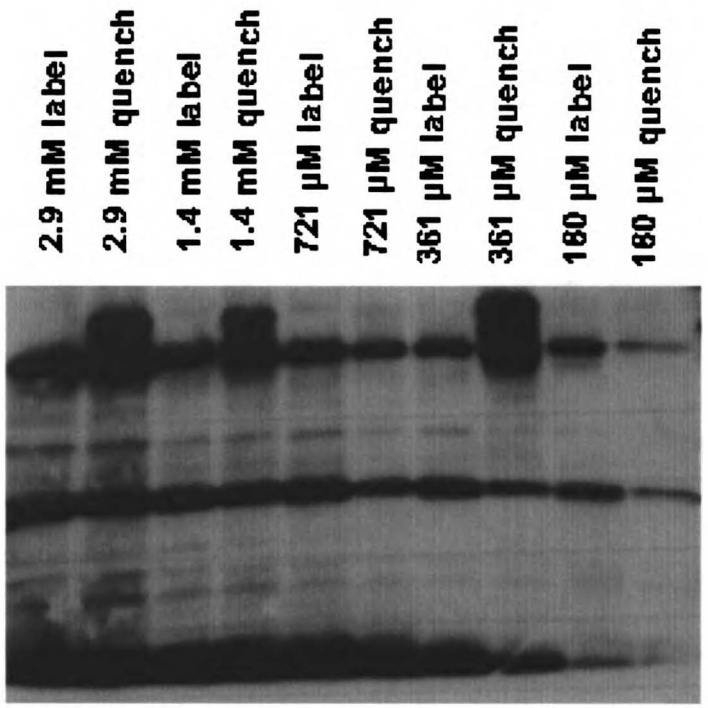

Figure 18. Alkoxyamine labeling in absence of enzyme

Figure 18 shows the alkoxyamine label incubated with HIP and BID proteins in the absence of granzyme B at concentrations of 180 µM to 2.9 mM. As seen in the avidin blot, significant labeling in observed. The nonspecific labeling increases with increasing label concentration.

Mass Spectrometry Characterization of N-terminal Labeled BID Product

In order to verify the peptide label is indeed covalently attached to the N-terminal product MALDI-TOF is conducted.

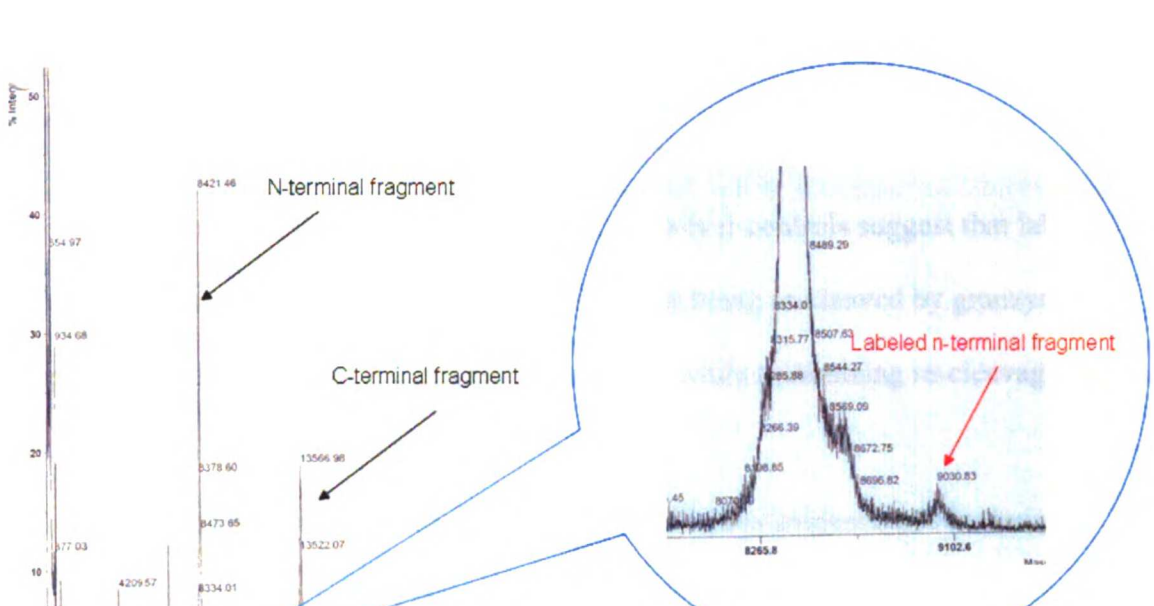

Figure 19. MALDI-TOF of labeled BID product

The MALDI spectrum (figure 19) shows the unmodified N-terminal BID product around 8.4 kDa and the C-terminal fragment around 13.6 kDa. The close up insert shows a peak around 9 kDa that is not present in the substrate plus enzyme control sample. This 9 kDa is the approximate size of the N-terminal products which has been modified by the nucleophilic label.

DISCUSSION

In this study nucleophilic tetrapeptides are synthesized, purified, and characterized. These peptide labels have been incorporated into the N-terminal fragment of various substrates. In order to observe N-terminal labeling, several conditions were established.

Time is an important parameter to consider. Naturally one is inclined to allow for longer periods to maximize proteolyzed product. However controls suggest that labeled product also serves as a substrate resulting in the label being re-cleaved by granzyme B. An optimal window between maximizing proteolysis while minimizing re-cleavage must therefore be established.

Samples using ecotin to quench active granzyme B show evidence of N-terminal labeling. However labeling of N-terminal product is predominantly seen when TFA is used to quench the reaction. This inactivates granzyme B in addition to protonating the label rendering it non-nucleophilic. Additional studies have shown that when substrate is incubated with label (S+L) and the pH is dropped, there appears to be some stripping of label from full-length substrate compared to a S+L sample kept at pH 8 (data not shown). This suggests that N-terminal labeling is acid-sensitive but not acid-dependent. Further characterization by LC-MS/MS is necessary in order to determine the exact location of the modification on the N-terminal fragment.

Another important consideration is the presence of unreacted label. Free label interferes with electrophoresis as well as analysis by mass spectrometry. In order to analyze labeling reactions, excess label must be removed through filtration.

A. Fluorogenic substrate labels versus biotinylated peptide labels

Preliminary studies indicate that the tag or reporter group is more important than originally anticipated. Use of the biotinylated peptide allowed higher concentration of label to be used without seeing inhibition of proteolysis compared to the fluorogenic labels. This is especially true when conducting assays in the absence of detergent. One explanation could be that the fluorophores form aggregates which sequester enzyme thus resulting in inhibition (McGovern,2003).

B. Protein engineering to increase specificity

The amino acid sequence of the nucleophilic label is chosen to match the preferred prime side sequence. A K41A/Y151A mutant granzyme B is engineered and various complementary knob labels are synthesized in hopes of increasing specificity. While quantitative studies have not been conducted, preliminary studies using knob labels with wild-type GrB versus the K41A/Y151A mutant indicates no difference (data not shown). This suggests that we have not created a unique interaction and that the labels are likely to interact with other enzymes. Further efforts with computational modeling may reveal better candidate sequences. It was hoped that the mutations in the prime side would not alter substrate specificity. However studies indicate that the mutations may have altered substrate recognition. Instead of cleaving BID once, when using the K41A/Y151A mutant, BID is cleaved twice resulting in two N-terminally labeled fragments.

C. Increasing the nucleophilicity of the peptide labels

The label has to compete with hydrolysis for a given number of turnover events. One goal of the study is to increase the reactivity of the nucleophilic label. Doing so would boost the amount of labeled product. There is an increased amount of non-specific labeling observed compared to using an amine as a nucleophile. The alkoxyamine labels may be useful in developing general labels for tagging proteins but appears to be too reactive to targeting a specific protease.

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