

Thimet Oligopeptidase: Fluorescent Labeling to Investigate Structural Changes

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The intent of the present study was to optimize procedures to monitor structural changes in the enzyme thimet oligopeptidase (TOP). Wild-type rat TOP was covalently labeled with the cysteine-specific, fluorescent probe N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD). Adding this fluorescent tag is analogous to adding an antennae for detection. This will allow structural changes to be monitored and analyzed under fluorescence spectroscopy due to the tag's sensitivity to the polarity of its environment.

The procedure done to modify the enzyme with IANBD involved covering surface cysteines with iodoacetate, leaving cysteines within the substrate binding cleft available for IANBD attachment. A tryptic digest and HPLC was done to isolate the peptide segment containing the fluorophore. The peptide fragments were eluted

and fractions were collected; currently they are awaiting mass spectrometry analysis at a facility in UCSF. Once the location of attachment is identified by mass spectrometry, further assays will be done to analyze the sensitivity of the fluorophore on site. Depending on the results, the enzyme will be mutated to rearrange the site of IANBD attachment to more plausible sensitive sites.

Activity assays were done and confirmed that the modification did not affect enzymatic performance. Ligand binding assays were also done with the inhibitor angiotensin (1-7) to detect initial changes in fluorescence when the enzyme is open or closed.

Conducting this research will provide more knowledge on the structure and function of TOP in an outcome to provide drugs and inhibitors that will bind strongly to the enzyme.

INTRODUCTION

Thimet oligopeptidase (TOP) (EC 3.4.24.15) is a metallo endopeptidase that is ubiquitous throughout the body with concentrated presence in the brain and gonads. It is homologous to several metallopeptidases across species of which all share a common HEXXH metal-binding motif¹⁻² i.e., a recurring structure. TOP functions to cleave an array of peptides which in effect involve immune responses and regulation of physiological activities such as lowering blood pressure and reproduction¹⁻². Another function that has intrigued further research is the enzyme's ability to degrade A β amyloid plaques present in Alzheimer's disease³.

X-ray diffraction studies have provided invaluable information with regards to its structure and confirmed structural homology to its closest relative, neurolysin¹⁻². Figure 1 shows the overall structure; TOP is primarily α -helical with two domains separated by a deep cleft where the substrate binds. Domain II contains the Zn(II) active-site and residues

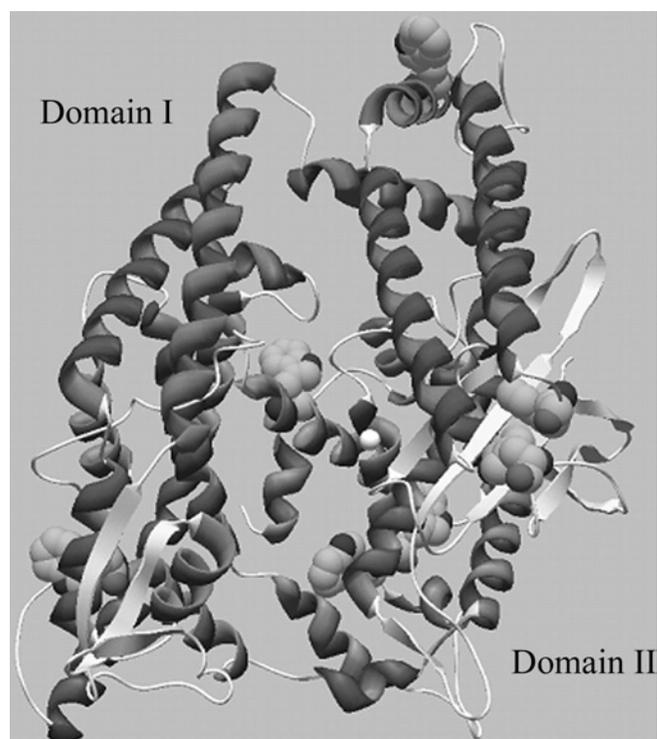


Figure 1¹: Overall tertiary structure of TOP, indicating the domains.

necessary for peptide cleavage. Domain I has a putative role for limiting substrate accessibility to the active site¹. Between the two domains, a hinge movement has been speculated due to the flexible loop regions connecting the domains. This movement poses curiosity with regards to substrate recognition, specificity and selectivity which can be connected to the function and properties of homologous enzymes.¹

In the present research, a method has been modified and produced to attach a fluorescent tag to the enzyme and to identify the location of attachment. The purpose of tagging the enzyme is to investigate and monitor structural changes during activity, particularly the hinge movement collaborated by the two domains.

This investigation will lead to better drug design and understanding of enzymatic activity. The fluorescent tag used was N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylene-diamine (IANBD); it is cysteine-specific and environmentally sensitive. The fluorescence of IANBD increases as polarity of its solvent environment decreases⁴. Figure 2 conveys this information; the fluorescence of IANBD changes in different solvents. Proteins that have been labeled can then be monitored by fluorescence spectroscopy to analyze conformational changes and protein-substrate interactions.

TOP has 14 cysteine residues, most of which

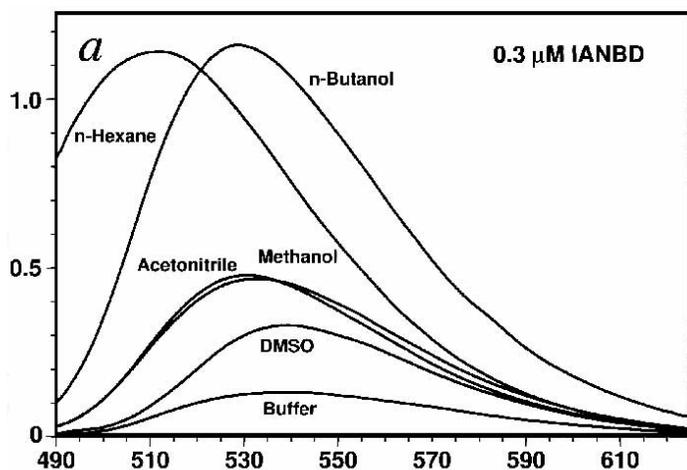


Figure 2^a: Fluorescence of IANBD in various solvents. This data from literature was referenced to the solvents IANBD was used in the assays for the set of experiments. The solvents used were acetonitrile and the prepared buffer in the Experimental Section. Both conditions were comparable to this data.

are located on the surface⁵. However, two residues, Cys175 and Cys425, are located within the substrate-binding cleft and are the target of attachment⁵. Tagging the enzyme will demonstrate if the sites are sensitive to structural changes when monitored by ligand binding assays. If the expected data and results are contrary, these two cysteines or other amino acids can be mutated into other residues to change the site of attachment to a more sensitive location.

EXPERIMENTAL PROCEDURES

Materials

Wild-type rat thimet oligopeptidase was provided by M.J. Glucksman (FUHS/Chicago Medical School, Midwest Proteome Center and Department of Biochemistry and Molecular Biology, Chicago, IL 60064, USA). The reductant tris(2-carboxyethyl) phosphine (TCEP) was obtained from Pierce. PD-10 columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). PepClean™ C-18 Spin Columns and In-Solution Tryptic Digestion and Guanidination Kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). The 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-Lys-dinitrophenol (MCA) fluorescent substrate was obtained from Bachem (King of Prussia, PA). Iodoacetate and N,N'-dimethyl-N-(iodoacetyl)-N'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine (IANBD) and all other reagents were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.).

Enzyme Modification

The following protocol was referenced from *Sigman et al., (2003)*⁵. A concentrated stock of wild-type rat TOP was obtained. A PD10 column was equilibrated with prepared buffer containing Tris-HCl and KCl with added reductant, TCEP (1mM), all at a pH of 7.8. Fractions of the enzyme were collected from the PD10 and checked via UV-vis. This step separated the DDT from the enzyme stock and transferred it into buffer with TCEP. The enzyme was then recombined from the fractions and concentrated in a centricon for 20mins at 4000rpm, then 1000rpm for one minute. A 50mM iodoacetate (IA) solution was prepared in 25mM phosphate and 5mM EDTA, all at a final pH of 8.03. The enzyme was retrieved from the

centricon and the concentration determined using its molar absorptivity $\epsilon_{280} = 73.11\text{mM}^{-1}\text{cm}^{-1}$, which was calculated based on the amino acid content of TOP using the automated ProtParam Tool on the SWISS ExpASy server¹.

An inhibitor solution was prepared using 100 μM of angiotensin 1-7 and added at twice the volume to maintain inhibitor concentration. The enzyme was incubated with the inhibitor for 5-10mins at room temperature; this kept the enzyme in a closed confirmation, blocking the inner cysteines from the IA reaction.

After incubation, an equivalent volume of IA was added to the enzyme and reacted for 90mins at 4°C then 15mins at RT. The PD10 column was re-equilibrated with buffer without TCEP and the reaction was run through to separate the excess IA solution. Fractions were collected and concentration determined as described above.

A 15mM IANBD solution was prepared. The volume of the modified enzyme was brought up to 400 μL . IANBD was titrated slowly to the enzyme and reacted at 4°C overnight.

The reaction was run through the PD10. The enzyme was concentrated down and stored at -80°C.

The stoichiometry of protein to IANBD was calculated using the molar absorptivity of IANBD at 481nm⁴.

Tryptic Digest

The procedure for the digest was based on the method provided from the kit, along with reagents used. Digestion and reducing buffers were added to 0.025-10 μg of modified enzyme and incubated at 95°C for 5 minutes. Alkylation buffer was added and reacted at room temperature for 20mins. The provided trypsin was added and incubated at 37°C for 3 hours. Another aliquot of trypsin was added and the reaction was incubated at 30°C overnight.

An optional guanidination procedure followed digestion; this step converted lysines into homoarginines to enhance downstream mass spectrometry analyses.

PepClean™ C-18 Spin Columns

To further purify the digested enzyme for

analysis, C-18 reversed-phase resin columns were used. The procedure used was provided by the kit. The washes from each step was collected and saved to undergo HPLC analysis.

HPLC Analysis

An Agilent HPLC 1200 series was used along with a size exclusion column. The mobile phase was 100% acetonitrile HPLC reagent grade. The flow rate was set to 0.500mL/min and peaks were detected at 481nm and 530nm by fluorescence along with the typical protein identifying wavelengths on the diode array detector.

Activity Assays

A fluorimeter was used to monitor enzyme activity; the instrument was Perkin Elmer Luminescence Spectrometer LS 50B. Activity assays were done to assess if modification of the enzyme affected activity. MCA was the substrate used at high and low concentrations. The assay required enzyme in prepared buffer described above. Activity was monitored under fluorescence spectroscopy at an excitation of 325nm and emission of 400nm.

Ligand Binding Assays

The ligand binding assays had similar requirements as the activity assays. Fluorescent changes were compared between IANBD-modified TOP with and without the inhibitor angiotensin(1-7). Changes were monitored under varying concentrations of enzyme and inhibitor. Excitation was at 481nm and emission wavelengths were monitored from 490nm to 625nm⁴

A summary of the procedure is depicted in Figure 3.

RESULTS & DISCUSSION

Optimization of HPLC usage was done extensively to obtain favorable separation of distinct peaks. Numerous buffers and flow rates were tested on various peptides such as MCA and various forms of MCA i.e., MCA-bradykinin. Running the peptides and undigested enzyme gave estimates on the retention time for the actual experiment. Figures 4 and 5 show

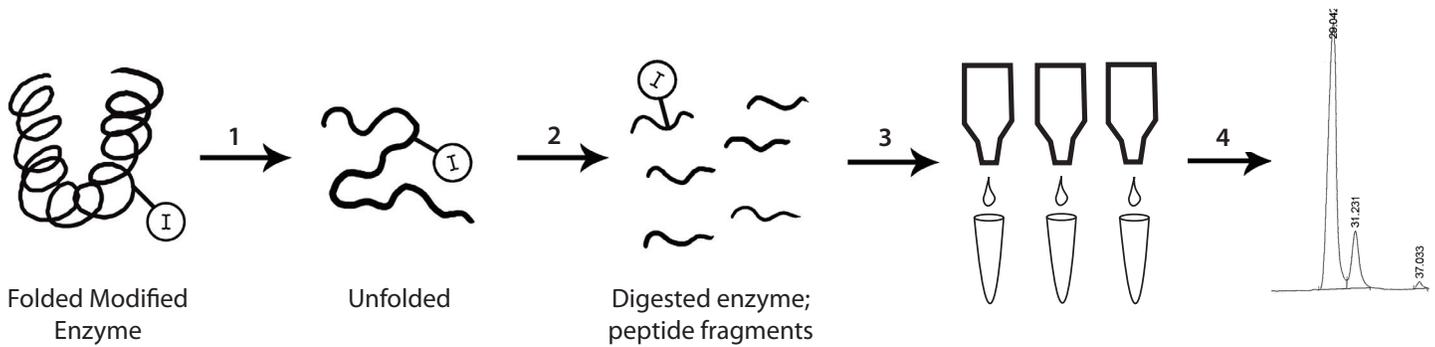


Figure 3: 1: Denaturation. 2: Digestion. The digest procedure was modified numerous times, to assure complete digestion of enzyme. Twice as much trypsin was added and overnight digestion was necessary. Also a higher amount (μg) of enzyme gave a better fluorescent intensity and compromised sample loss downstream. 3: Downstream purification. 4: HPLC. The chromatograph is enlarged in Figure 8.

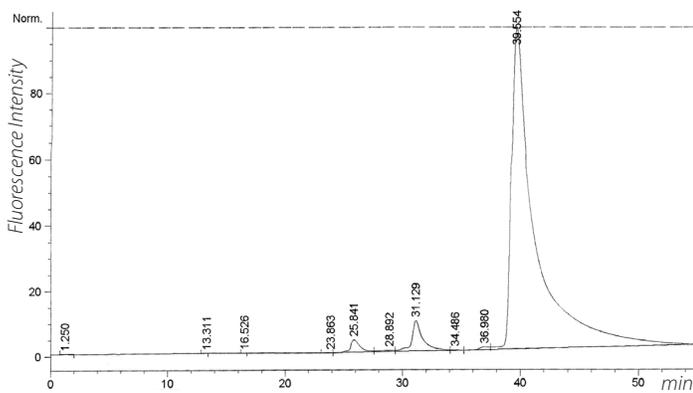


Figure 4: HPLC chromatogram of MCA-GnRH (Gonadotropin releasing hormone attached to MCA for fluorescent properties). After numerous sample runs, a flow rate of 0.500m/min in acetonitrile gave the best results. This data shows that peptide fragments were estimated to have a retention time of ~ 30 mins when running the digested TOP.

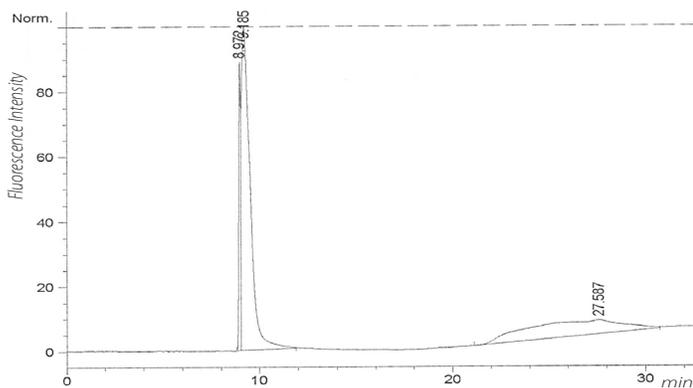


Figure 5: HPLC chromatogram of undigested, modified TOP. This data is a reference to running the digested TOP, if there is undigested enzyme left, the digest procedure needs to be modified by adding more trypsin or longer incubation period. The retention time was ~ 10 mins. The retention time shown at 27.587min is likely contamination.

HPLC chromatograms of a peptide and undigested enzyme.

Evidence of enzyme modification was given by UV-vis spectroscopy. Figure 6 shows the spectra of TOP before and after modification. Activity assays were then done to verify that the modification did not affect enzymatic performance; Figure 7 depicts this. All enzyme concentrations were kept at $0.1 \mu\text{M}$. The substrate concentration used for WT and IA was $40.688 \mu\text{M}$ and $81.376 \mu\text{M}$ for IANBD-TOP. In the graph, it shows that the activity of IANBD-TOP is twice as much than WT-TOP due to the difference of substrate concentration. However the activity of IA-TOP increased compared to WT-TOP. This is most likely due to dimerized enzyme in WT-TOP⁵ where it would not be a problem for IA-TOP since the cysteine residues are covered.

HPLC analysis followed the tryptic digest and samples were run to determine retention times of the peptide fragments. The results are shown on Figure 8. Fractions at the resulting retention times were collected. About 20-25 fractions were collected over the period of time the HPLC was running. The fractions were sampled under fluorescence spectrometry to locate the fluorescent tag. Wherever the tag was located via fluorimetry, the fraction was set aside to be sent for mass spectrophotometry analysis.

Ligand binding assays were then done to observe changes in fluorescence intensity when the enzyme would open and close. An initial change was seen in sample 1 with $125 \mu\text{M}$ angiotensin(1-7). This is depicted as the red curve in Figure 9. This complied to the characteristic of IANBD; fluorescent intensity increases as polarity of solvent decreases. There must

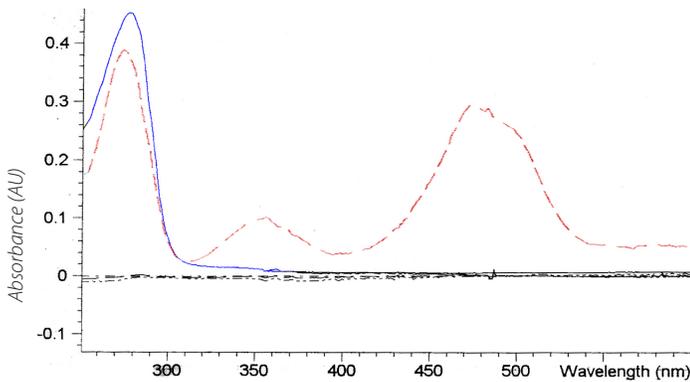


Figure 6: UV-vis spectra of TOP before and after modification. The blue peak curve represents unmodified TOP at 280nm. The red curve is modified TOP. The peak at 350nm is iodoacetate and IANBD at 481nm.

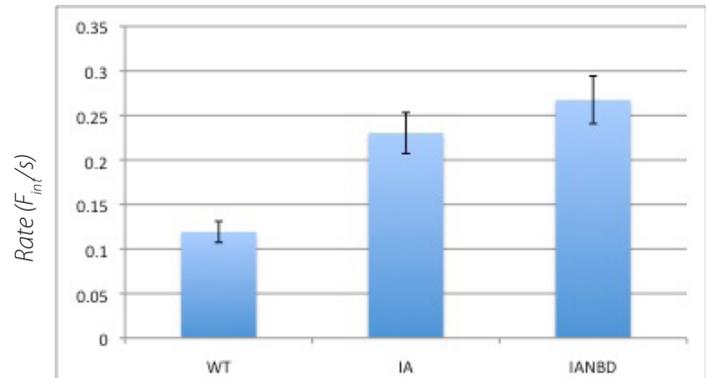


Figure 7: Activity rate comparison of TOP at different stages of modification. WT for wild-type state. IA for iodoacetate attachment to TOP. And IANBD for the final step.

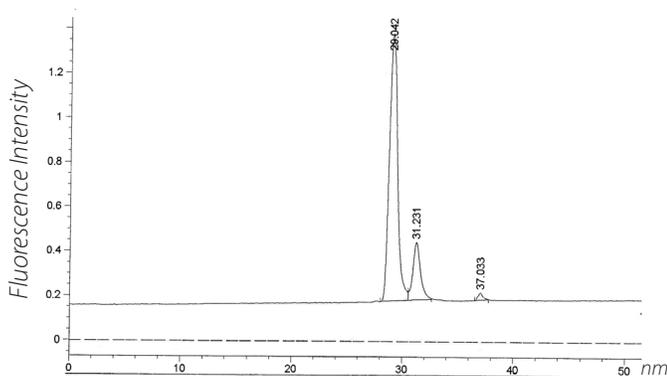


Figure 8: Three prominent peaks were seen after running multiple samples for consistency. The major retention times were these three as shown: 29.042mins, 31.231mins, and 37.033 minutes.

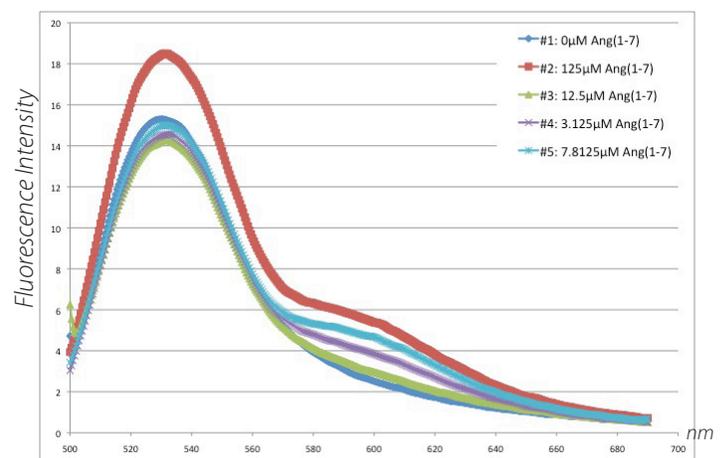


Figure 9: Ligand Binding Assays of wild-type TOP modified with IANBD (WT-IANBD). 0.25µM was the initial concentration of enzyme used and inhibitor (Ang (1-7)) was titrated.

have been an increase in fluorescence because TOP is bound to angiotensin(1-7), which is a competitive inhibitor. As angiotensin was bound, TOP was in a closed conformation, pushing out solvent from the tag and caused a change in the fluorescence of IANBD. Another observation seen was a leg in the curve forming as opposed to trailing off to baseline which was the case in WT-IANBD without angiotensin. Samples following this varied in both substrate and enzyme concentrations. The trend seen was a decrease in fluorescence to an intensity similar to WT-IANBD without inhibitor. However the leg in the curve was still present.

Currently the identification of the peptide fragment bearing the fluorescent tag is awaiting results from mass spectrometry from the facility in UCSF.

CONCLUSION

A method has been developed to successfully modify TOP with a fluorescent tag without altering enzyme functional integrity. The assays done in assessment of the enzyme needs to be repeated for further consistency. Different substrates can also be used for the ligand binding assays to observe if they have various affects in fluorescence intensity. Any changes in intensity convey if TOP undergoes conformational changes. Data and information obtained from this experiment can also be used to assess relative enzymes across species, particularly neurolysin.

Once results from mass spectrometry are received, more information will be provided on the location of the tag and structural changes in relation to where the tag is attached. Further experiments will be done as necessary.

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