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LC-MS Reveals Isomeric Inhibition of Proteolysis by Lysosomal Cathepsins

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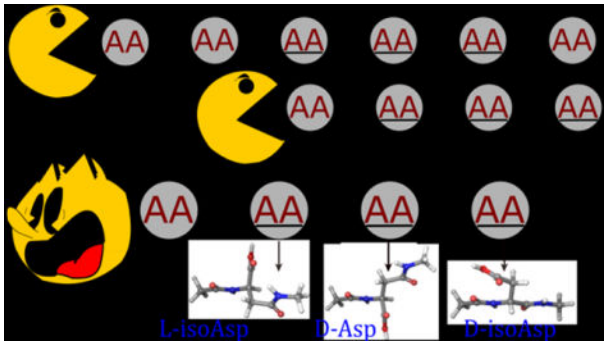
Abstract

Defects in autophagy are implicated in many age-related diseases that cause neurodegeneration including both Alzheimer's and Parkinson's. Within autophagy, the lysosome plays a crucial role by enabling the breakdown and recycling of a wide range of biomolecular species. Herein, the effects of isomerization of aspartic acid (Asp) on substrate recognition and degradation are investigated for a collection of lysosomal cathepsins using liquid chromatography coupled to mass spectrometry. By examining a series of synthetic peptides with sequences derived from long-lived proteins known to undergo Asp isomerization, we demonstrate that isomerized forms of Asp significantly perturb cathepsin activity by impeding digestion and shifting preferential sites of proteolysis. Although the sensitivity to isomerization varies for each cathepsin, none of the cathepsins were capable of digesting sites within several residues of the C-terminal side of the isomerized Asp. Under physiological conditions, the peptide fragments left behind after such incomplete digestion would not be suitable substrates for transporter recognition and could precipitate autophagic malfunction in the form of lysosomal storage.

Graphical Abstract



Keywords

Alzheimer's disease; LC-MS; isomerization; long-lived proteins; proteomics

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Introduction

Research in the past decade has generated extensive evidence highlighting the crucial role of dysfunctional lysosomes in Alzheimer's disease (AD).[1] Lysosomes, also known as the scavengers of the cell, are organelles that digest intracellular macromolecules into constituent components. They play a critical role in the process of autophagy, which in turn is essential for maintaining protein homeostasis or proteostasis (i.e. the balance between protein synthesis and degradation).[2] In macroautophagy (hereafter autophagy), non-functional, misfolded, or aggregated proteins are transported to lysosomes, where proteolytic enzymes degrade the cargo.[2] Endopeptidases hydrolyze proteins at internal sites, reducing proteins to peptides, which are subsequently digested from the C- and N-termini by carboxy- and amino-peptidases, respectively. After complete breakdown, transporter enzymes in lysosomal membranes shuttle individual amino acids back into the cytosol for the next round of protein synthesis or energy generation. Defects in autophagy associated with AD include the observation of lysosomal storage comparable to that observed in lysosomal storage disorders (LSDs). Moreover, cerebrospinal fluid samples of patients with Niemann-Pick type C (a LSD) have been reported to have high levels of γ-secretase-dependent Aβ, a known pathological feature of AD.[3]

These observations suggest an intersection between the pathologies leading to AD and LSDs. In LSDs, aberrant mutations to genes encoding lysosomal hydrolases represent the most common underlying cause. This leads to incomplete substrate digestion, accumulation, and ultimately lysosomal storage. The abnormal build-up of failed lysosomal bodies is especially antagonistic for postmitotic cells like neurons, where storage cannot be ameliorated by cell division.[4] As a consequence, LSDs are often associated with progressive neurodegeneration and premature death.[5] Although similar pathology is observable in AD, the underlying cause is not the same, but it has been suggested recently that an inability to degrade isomerized substrates could be a significant contributing factor.[6]

Isomerization is known to occur in long-lived proteins (LLPs), particularly at aspartic acid (Asp) residues by formation of a succinimide ring, which can subsequently undergo hydration yielding L-Asp or L-isoAsp (Scheme 1).[7,8 9,10] The L-succinimide intermediate can also flip chirality to form D-Asp or D-isoAsp, ultimately leading to population of four different isomeric forms. Additionally, Asn can undergo deamidation and convert to the same four Asp isomers.[11] In general, the rate of Asn deamidation is faster than Asp isomerization, but recent results have shown that Asp in Aβ can rapidly isomerize at nearly 1% per day.[6] The structural changes caused by Asp isomerization impede proper binding within the catalytic sites in enzymes and can disrupt proteolysis in the case of proteases.[12] The negative charge introduced by deamidation can also impact the structural integrity of the proteins.[13] In the case of either L/D-isoAsp, addition of a backbone methylene can adversely affect the conformation, stability, and activity of the native form by creating a 'kink or bend' distorting the protein chain.[14] Isomerization is one of many non-enzymatic, age-dependent alterations that comprise spontaneous chemical modifications (SCMs), which are significant contributors to protein deterioration and exist largely outside enzymatic control.[15] In addition to AD,[16,17] SCMs are associated with aging,[18] eye lens abnormalities[19,20] and type 2 diabetes.[21]

In vivo accumulation of isomerized Asp is highly toxic. Hence, a repair mechanism driven by a ubiquitously expressed enzyme, protein-isoaspartyl methyl transferase (PIMT), is in place to negate its consequences.[22] PIMT selectively catalyzes the repair of the L-isoAsp by methylation of the unnatural side chain carboxylic acid. This facilitates rapid formation of the succinimidyl intermediate, which can convert back to the L-Asp form by non-enzymatic hydrolysis (Scheme 1).[23] Although PIMT-initiated restoration is not entirely efficient (i.e. the primary product following methylation equals the initial substrate),[24] disruption of PIMT activity leads to fatal epilepsy in transgenic mice.[25,26] PIMT function therefore appears to be especially critical to neurons in mammals. Moreover, the deletion of PIMT coding genes in bacterial and eukaryotic systems generates aberrant phenotypes akin to a stress-induced state.[27,28]

The long-lived proteins tau and Aβ contain multiple Asp residues known to isomerize in vivo,[29–32] but it is not known whether digestion of these species can be effectively carried out by the cathepsins (Cat), which serve as the primary proteases within lysosomes.[33] Some previous work has demonstrated that isomerization can impede proteolysis in general,[34–36] but minimal work has been done with lysosomal proteases.[6,37,38] In a recently published study from our group, we demonstrated that isomerization and epimerization of Asp or Ser residues in peptides impedes digestion for certain cathepsins and peptides,[6] but much remains to be examined about the full range of cathepsin interactions with isomerized peptides.

In the present work, we combine liquid chromatography (LC) with mass spectrometry (MS) to characterize the impact of isomerized aspartic acid residues on the activity of a representative suite of lysosomal cathepsins. Target substrates include biologically relevant peptide segments from long-lived proteins in the brain and eye where aspartic acid has previously been found to spontaneously isomerize. The assay results show that peptides containing isomerized versions of Asp, resist both endo- and exopeptidase action of cathepsins at preferred sites. The samples containing isomerized residue show increased levels of undigested precursor. The level of proteolytic degradation varies across different enzymes, and the sites of proteolysis are shifted. In addition, some endopeptidase activity is observed in enzymes primarily known for their exopeptidase mode of action.

Results and Discussion

There are 15 known human cathepsins that utilize one of three classes of active sites, cysteine (CatL, V, K, S, H, B, F, W, C, O, Z), aspartic acid (CatE, D), and serine (CatA, G).[39] The prevalence and importance of each cathepsin within the lysosome vary substantially and are in some cases poorly explored.[33] Cathepsins selected for this study include B, L, H, F, C, and A (Table S1). CatB and L are among the most abundant proteases in the lysosome and are vital for cellular health through exertion of endopeptidase activity with broad specificity. Cathepsin F represents a novel, more recently discovered class of cathepsin, with potentially novel substrate specificity.[40] CatC cleaves dipeptides from the N-terminus, a unique cleavage preference among the cathepsins. CatA represents the only example of a serine carboxypeptidase among the cathepsins. The remaining cathepsins were not included in this study for reasons as follow. CatD and CatH were examined previously

for isomer degradation using the same protocol,[6] and CatE shares high structural and cleavage preference with CatD.[41, 42] CatV, S, and K share high structural, sequence, and substrate specificity with CatL. CatW shares sequence similarity to CatF and is not known to be vital for lysosomal function. CatO and CatZ are cysteine proteases, for which several more prominent examples will be studied. CatG cleaves at an optimal pH of 8, making it unlikely to be critical for lysosomal function.[43]

For substrate selection, we looked to known examples of isomerized sites in long-lived proteins. For example, Asp 387 of tau is known to be isomerized in samples derived from AD patients.[31,44,45] Therefore, the peptide segment AKAKTDHGAEIVYK (tau 382–395), derived from human tau protein, was chosen as the primary test substrate. The canonical peptide and its Asp variants were proteolytically digested in isolation with each of the cathepsins detailed above. The impact of isomeric modifications on the activity of the proteases during the incubation period was examined by relative quantification of the proteolytic fragments from the aliquots collected at various time points. Based on the area of each LC-MS chromatogram peak detected in each sample, the fractional abundance (FA) of each fragment was calculated by using the following equation:

$$FA = \frac{Area\ of\ the\ LC\ peak}{\sum Area\ of\ all\ peaks} \times 100$$

Cathepsin L is one of the most aggressive lysosomal proteases, suggesting it may have the potential to digest isomerized sites. Enzymatic hydrolysis of the canonical version of AKAKTDHGAEIVYK with cathepsin L in acetate buffer at pH 5 yielded numerous peptide fragments, as illustrated in Fig. 1. Each data point in Fig. 1 represents the fractional abundance for one peptide at one time point as derived from LC-MS data. The results are binned by fragment (where background color shifts highlight different peptide fragments), and time points increase in length from left to right for each peptide. High points in Fig. 1 correspond to higher abundance of the peptide fragment. The different colored lines represent different isomeric forms of the peptide. This same type of plot will be used to represent most of the digestion data in this manuscript. The leftmost blue column illustrates data for the full-length peptide precursor, AKAKTDHGAEIVYK. Although all isomeric forms are degraded by CatL, the canonical form depletes more rapidly, particularly at early time points. The IVYK fragment, which is remote from the site of isomerization, is generated fairly consistently for all isomers. In contrast, cleavage to generate TDHGAEIVY is favored for the canonical peptide, but the proximity of the isomerized residue appears to interfere with cleavage for all isomerized forms. Similar results are observed for the HGAEIVYK and GAEIVYK fragments, which are cleavages just C-terminal to the isomerized asp.

CatL prefers basic and hydrophobic amino acids at P1 and P3 positions (according to the Schechter and Berger nomenclature shown in Fig. S1[46]), respectively.[47,48] The fragments derived from cleavages at preferred sites away from the isomerization site (IVYK, AKTDHGAEIVYK, AKAKTDHGAEIVY, IVY) showed comparable fractional abundances across all the isomeric forms. In contrast, cleavage near Asp only occurred in the canonical form, indicating that isomerized Asp in this crucial Tau fragment impedes degradation by

CatL. These results are consistent with previous experiments[6] examining the effects of isomerization on digestion by CatL, providing further evidence that proteolytic impairment likely to persist regardless of local sequence.

Data for the analogous experiment with cathepsin A, a monocarboxy peptidase, in MES buffer, pH 6.0 are shown in Fig. 2. The leftmost section of Fig.2 shows eventual precursor depletion for all four isomeric versions, although the precursor is completely consumed for the all-L peptide within 2 hours of incubation. In contrast, the isomerized-Asp-containing peptides exhibit prolonged precursor survival, suggesting less affinity toward proteolysis in general. The subsequent columns show fractional abundances of N-terminal fragments AKAKTDHGAEIVY, AKAKTDHGAEIV, and AKAKTDHGAEI deriving from sequential losses of C-terminal residues (as expected for CatA). The fractional abundance of the AKAKTDHGAEIVY peak in the all-L sample is comparable to the non-canonical peptides at t=0.5h, however, it is rapidly digested, presumably yielding the AKAKTDHGAEIV fragment. The isomeric forms of AKAKTDHGAEIVY undergo slower digestion, accounting for the delayed increase in the fractional abundance of AKAKTDHGAEIV. AKAKTDHGAEI intensity also increased slowly, yielding markedly lower values for the non-canonical variants. Overall, it is clear that the all-L peptide is more prone to enzymatic hydrolysis while the isomerized samples resist the exopeptidase activity. Interestingly, multiple internal fragments (KTDHGAEIV, AKTDHGAEIV, KTDHGAEI, AKTDHGAEI) were also observed in the canonical digest, revealing a degree of endopeptidase activity for CatA that has not been previously reported. Distinctively, no internal fragments were observed in the non-canonical samples, indicating that a single isomeric modification modulates substrate recognition and completely halts endopeptidase action. In addition, it appears that glutamic acid may interfere with CatA digestion due to reluctant degradation of the canonical peptide in proximity to glutamic acid,[49] although this effect is secondary to that exerted by isomerized Asp.

Data for CatC, a di-amino peptidase, collected over a 24-hour period are shown in Fig. 3. The native peptide precursor is reduced to around 50% after 24 hours of enzymatic digestion. For the non-canonical peptides, L-iso- and D-Asp containing peptides show a considerably higher amount of residual precursor, indicating suppressed exopeptidase activity. However, the D-isoAsp form showed slightly higher levels of precursor degradation compared to the canonical sample, suggesting enhanced association with the enzyme active site. The fractional abundance of AKTDHGAEIVYK originating from loss of AK maps complementarily with precursor depletion. The proteolytic activity of the enzyme is unchecked in the native peptide, and it proceeds to make the next dipeptide cut, yielding the TDHGAEIVYK fragment with increasing abundance over time. Proteolysis by CatC is impeded near the modified Asp in each of the non-canonical peptides, and no TDHGAEIVYK fragment is observed, suggesting the enzyme is unable to gain access to the isomeric site. Interestingly, in the LC-MS data for the D-isoAsp sample (which initially showed higher susceptibility to CatC), we also observed IVYK, confirming the less reported and less discussed endopeptidase activity of the enzyme.[50] CatC is known to prefer substrates that have lysine or arginine residues at the P1 position.[51] Failure to generate TDHGAEIVYK for the isomerized forms demonstrates that the presence of isomeric Asp can completely shut down proteolysis at an otherwise preferred site.

Results for analogous assay conducted in acetate buffer at pH 5 with CatH are compiled in Fig. 4. CatH is primarily a mono-amino peptidase, though it is also known to exhibit endopeptidase activity, where it prefers hydrophobic residues at P2/P3 positions.[52] Examining the data in the leftmost bin of Fig.4 reveals rapid precursor digestion in each of the four samples, although D-Asp appears most favorable. The following four bins in the plot highlight N-terminal exopeptidic cleavages and comparable digestion profiles, indicating that the N-terminal region is accessible regardless of isomeric form. Interestingly, the D-Asp form continues to show a slightly higher susceptibility to CatH. CatH continues cleaving N-terminal residues to within one amino acid of the isomerized Asp, yielding the TDHGAEIVYK fragment. This behavior contrasts that observed for CatC, which was unable to approach the isomerized site, perhaps because CatH is mono-aminopeptidase and does not need to accommodate two amino acids in the active site.

For the all-L peptide, CatH continues to make sequential cleavages, reducing it to DHGAEIVYK segment. For isomerized Asp forms, DHGAEIVYK is barely observed, and no further exopeptidic cleavages were observed past the isomerized sites. The all-L, D-Asp and the D-isoAsp containing samples also yielded IVYK albeit at very low fractional abundance, generated from endopeptidase cleavage around Glu-10 aided by the hydrophobic Tyr-13 at the P3 position. These results further confirm that CatH is incapable of digesting peptide bonds near isomerized residues.[6]

An identical analysis was performed after incubating the canonical and non-canonical versions of the AKAKTDHGAEIVYK peptide with cathepsin B, both an endo and a di-carboxypeptidase. The data reveal rapid precursor digestion and removal of the hydrophobic residues for all four isomeric forms as shown in Fig. S2. The remaining peptide fragments that contain the site of isomerization are poorly retained, complicating direct observation of the effects of Asp isomerization on proteolysis. To resolve this complication, an analogous assay was conducted with the all-L, L-isoAsp, D-Asp, and the D-isoAsp versions of SEMRLEKDRFSVNL, a peptide derived from αB-crystallin,[53] an important long-lived protein in the eye lens. The results are shown in Fig. 5. The leftmost bin shows that the precursors for the L-Asp and the D-Asp forms of the peptide are rapidly digested, however, the L-iso- and the D-isoAsp peptides are more resistant for up to 8 hours of digestion. The subsequent column shows the data for the SEMRLEKDRFS fragment. The all-L form of SEMRLEKDRFS is very short-lived, forming at t=0.5 and disappearing in the following time points. In contrast, the isomeric variants exhibit a gradual reduction in fractional abundance. The D-Asp and the D-isoAsp versions of SEMRLEKDRF and SEMRLEKDR exhibit higher fractional abundance compared to the L-Asp and the L-isoAsp forms. The data also reveal that digestion of the all-L peptide yielded higher levels of short endopeptidic fragments (VNL, NL, LEKDRF, EKDRF). The rightmost bin shows the fractional abundance of the KDRF, for which the L-isoAsp sample shows considerably higher fractional abundance relative to the other two non-canonical variants. The increased susceptibility of the L-iso form to CatB proteolysis can be attributed to the enzyme recognizing the α-carboxylic acid of the L-isoAsp as a carboxy-terminus of amino acid.[54] In general, the differences between the digestion profiles of the canonical and the non-canonical forms are smaller for CatB, highlighting the aggressive proteolytic activity of cathepsin B. However, digestion immediately C-terminal to any isomerized site is not

observed, which would be required to clear any of the non-canonical peptides from the lysosome.

The LC-MS acquired chromatograms for cathepsin F digestion of the AKAKTDHGAEIVYK peptide show no proteolytic digestion for the canonical or modified peptides, indicating unfavorable binding and high substrate specificity of the enzyme (Fig. S3). Hence, an analogous assay was conducted with the all-L, L-isoAsp, D-Asp, and the D-isoAsp versions of SEMRLEKDRFSVNL. The results reveal reluctant, though detectable, digestion for this sequence as well (see Fig. 6). The native peptide is reduced to ~70% after 24 hours digestion, yielding considerable amounts of SEMRLE and KDRFSVNL. A small amount of SEMR was also detected at longer time-points. Substitution of L-Asp with isomeric forms of Asp renders the peptide completely impervious to CatF digestion, even after 24 hours of incubation. Therefore, a single residue modification blocks the peptide hydrolysis and checks cathepsin F from hydrolyzing an entire 14 residue peptide.

Conclusion

Our results show that isomerization of a single amino acid perturbs proteolysis for a wide range of cathepsins. Both endo- and exopeptidase activity are impacted. In the majority of cases, proximity to an isomerized residue reduces proteolytic cleavage, although we also observed enhanced cleavage at sites remote to the isomerized residue in a few instances. Importantly, neither cleavage C-terminal to L-isoAsp nor liberation of an isomerized amino acid was observed in any of our experiments. Therefore, short peptide fragments would be expected to persist within the lysosome and could potentially contribute to lysosomal storage over extended periods of time. Our observations can be most easily rationalized based on inherent incompatibility between isomerized residues and catalytic active sites within proteases. Although amino acid substitutions can alter the sequence specificities between proteases, isomerization places side chains or the peptide backbone (or both) in unexpected locations that appear to be incompatible with proteolytic degradation.

Experimental

Peptide Synthesis:

Two 14-mer peptide segments, AKAKTDHGAEIVYK, (derived from canonical tau), and SEMRLEKDRFSVNL (derived from small heat shock protein in the eye lens αB-crystallin) and their L-isoAsp, D-Asp and the D-isoAsp variants were synthesized following an accelerated FMOC-protected solid-phase peptide synthesis protocol.[55] Isomerized peptides were synthesized by incorporating the appropriate isomeric form of the amino acid residue during synthesis. Following synthesis, peptides were purified on a Thermo Fisher Ultimate 3000 uHPLC using a Phenomenex Kinetex Evo C18 5 μm 100 Å 250 mm × 21.2 mm column using water with 0.1% formic acid for mobile phase A, and acetonitrile with 0.1% formic acid for mobile phase B with a flow rate: 18 ml min^{-1} and injection volume 2 mL. The gradient used for the purification is listed in Table S2. Post HPLC purification the mass of the peptides was validated by LC/MS. Purified peptides were stored frozen in 50/50 acetonitrile/water (v/v) and vacuumdried before reconstitution in appropriate buffers.

Cathepsin Digestion Assay:

Aliquots containing 50 μg of purified AKAKTDHGAEIVYK and SEMRLEKDRFSVNL were taken. Digestions with cysteine cathepsins (CatB, CatF, CatH, CatL) were performed in 50 mM acetate buffer pH 5 with 1 mM Ethylenediaminetetraacetic acid (EDTA) and 1 mM Dithiothreitol (DTT) to prevent active site oxidation. Pro- Cathepsin A and C were supplied as 'ProCathepsins' from the manufacturer (R & D Systems, Minneapolis, MN). The Pro-Cathepsins or the inactive zymogen versions of CatA and C were proteolytically activated to their mature form using the manufacturer recommended protocol. Following the manufacturer's suggestion, 2-(NMorpholino) ethanesulfonic acid hydrate buffer (MES buffer) was used in CatA and CatC assays. MES buffer is one of the most extensively non-coordinating buffer buffers used in biological assays. Cathepsin A, a serine protease, was activated by incubating with a cysteine protease, CatL, in a 1:4 ratio in an activation buffer comprising 25 mM MES buffer, pH 6.0, and 5 mM DTT, at 37°C, for 1 hour. The reaction was halted by deactivating CatL by incubating with 10 μM L-trans-Epoxysuccinyl-leucylamido(4-guanidino) butane (E-64), a cysteine peptidases inhibitor, at 37°C for 70 minutes. E-64, an epoxysuccinyl derivative is known to irreversibly inhibit the cysteine proteases (structure shown in Fig. S4).[56] Pro-Cathepsin C was also activated by incubating with CatL. However, a much-reduced amount (1:100) of CatL was used to avoid CatL interference. CatC is also a cysteine protease and is susceptible to E-64 inhibition. Although literature states that the E-64 inhibits CatC, albeit only at high concentrations ~500 μM,[57] we observed the activity inhibition at much-reduced concentration. Hence E64-mediated inhibition of CatL was avoided in the latter case. Post activation, the digestions with CatA and CatC were performed in assay buffer comprising 25 mM MES buffer, pH 6.0, 50 mM NaCl, and 5 mM DTT, at 37°C. The enzyme ratios were adjusted to maximize the number of peptide fragments that could be observed within the experimental timeframe.; CatL and CatB were used at an enzyme: substrate ratio of 1:150, CatA at 1:100, CatC at 1:50, CatF, and CatH at 1:25. In the case of SEMRLEKDRFSVNL peptide digestion with CatB, the enzyme: substrate ratio was 1:75. Time point aliquots were taken at 0.5, 2, 4, 6, 8 hours for cathepsins L, A, B, while an additional 24-hour time point was collected for cathepsins C, H, and F, as they showed slower rates of proteolysis. The aliquots were diluted to a final concentration of 5 μM with 0.2% TFA solution to pH quench the enzymatic reaction. Digested samples were frozen until analysis.

LC-MS Analysis:

A combined liquid-chromatography-mass-spectrometry (LC-MS) approach was used to evaluate protease efficiency. The samples were analyzed on an Agilent 1100 HPLC interfaced with a Thermo Fisher LTQ or a Thermo Fisher Ultimate 3000 RSLCnano System interfaced with a Thermo Fisher Velos Pro Orbitrap using electrospray ionization (ESI) sources. For the Agilent 1100, peptides (sample volume 50 μL) were separated on a Thermo Scientific BetaBasic C-18 3 μm, 150 × 2.1 mm column using water with 0.1% formic acid for mobile phase A, and acetonitrile with 0.1% formic acid for mobile phase B with a flow rate: 0.2 ml min^{-1}. The Ultimate 3000 was operated with a capillary column packed in-house to a length of ~20 cm with Waters Acuity CSH C18 3 μm resin using a Shotgun Proteomics Inc high-pressure vessel. For the Ultimate 3000, 1.5 μL sample volume and the same mobile phase A and mobile phase B were used with a flow rate of 0.3 μl min^{-1}. The

gradient used for the LC separations is listed in Table S3. Based on replicate datasets, the average error + 2 standard deviations is roughly 5% of the fractional abundance. Variations outside this range are likely to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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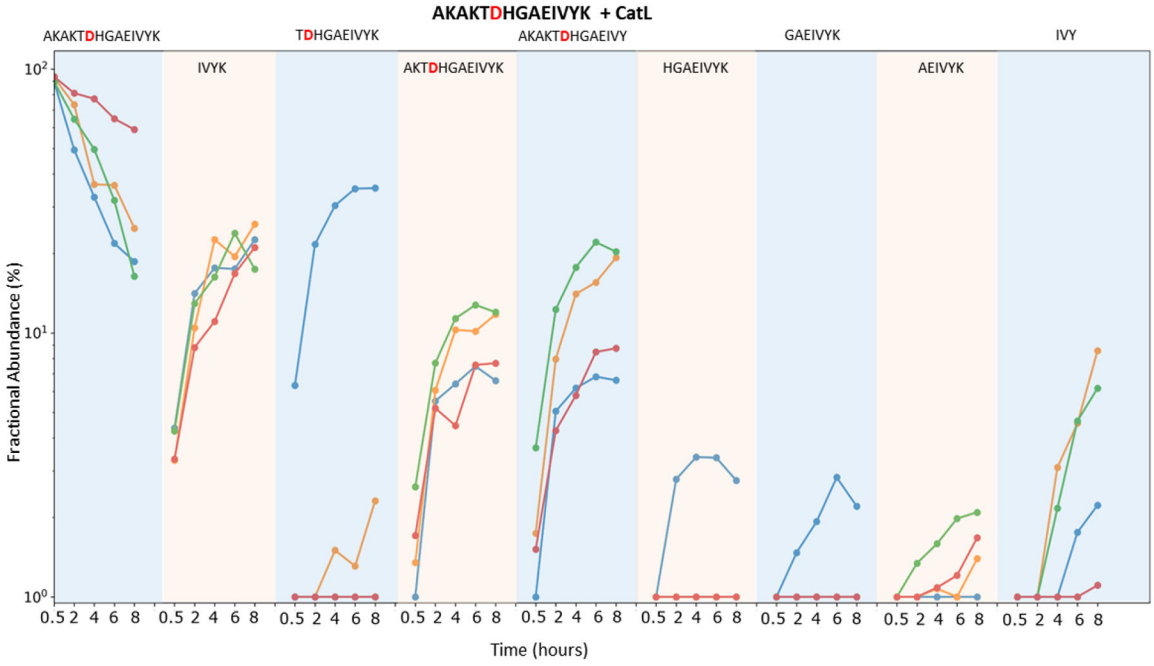


Figure 1.

Graph showing the $\log_{10}$ fractional abundance of the proteolytic fragments at various time points. Results are binned by peptide fragment, with color shading to highlight transitions between different fragments. The results for each isomeric variant are represented by a single colored line. In general, the L-Asp (blue) precursor depletes more quickly (far left) than isomers, and L-Asp populates fragments with cleavages near Asp much more than other isomers.

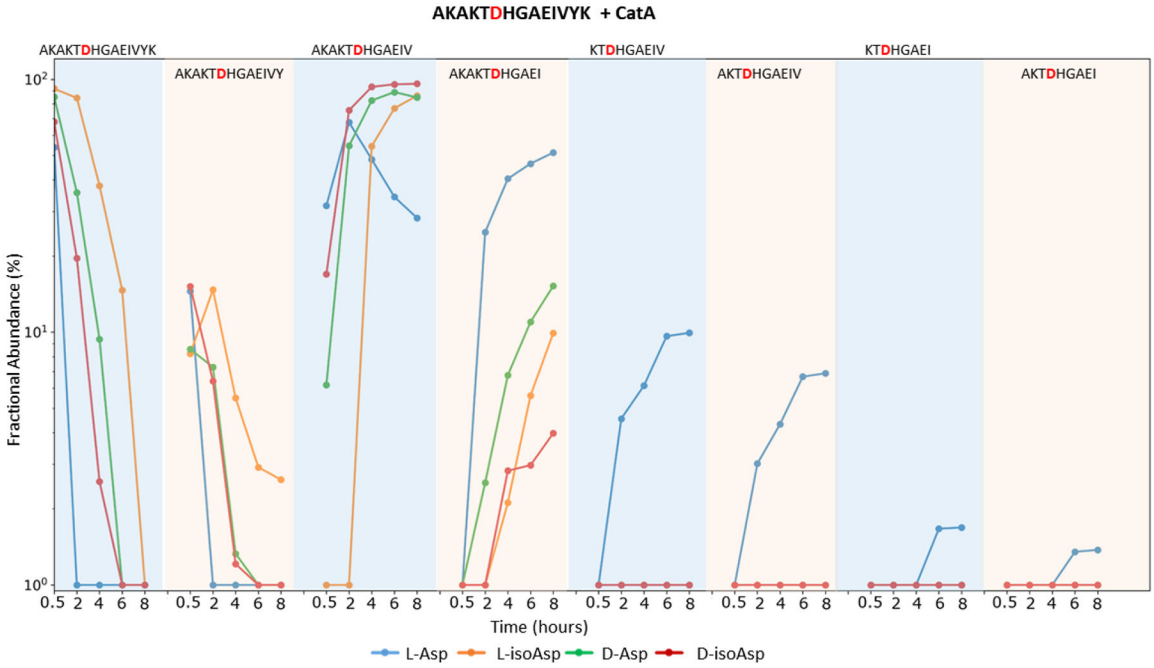


Figure 2.

Data is plotted for digestion of AKAKTDHAGEIVYK by cathepsin A using the same format outlined for Fig. 1. The digestion of the all-L peptide with CatA yields rapid precursor consumption and a few N-terminal fragments derived from the exopeptidase action. In addition, several internal fragments were also found, revealing the endopeptidase potential of the enzyme. In contrast, L-iso-Asp, D-Asp and D-isoAsp variants exhibit slower rates of precursor reduction and show no internal cleavages, demonstrating these modifications reduce the exopeptidase activity and entirely halt the endopeptidase action of CatA.

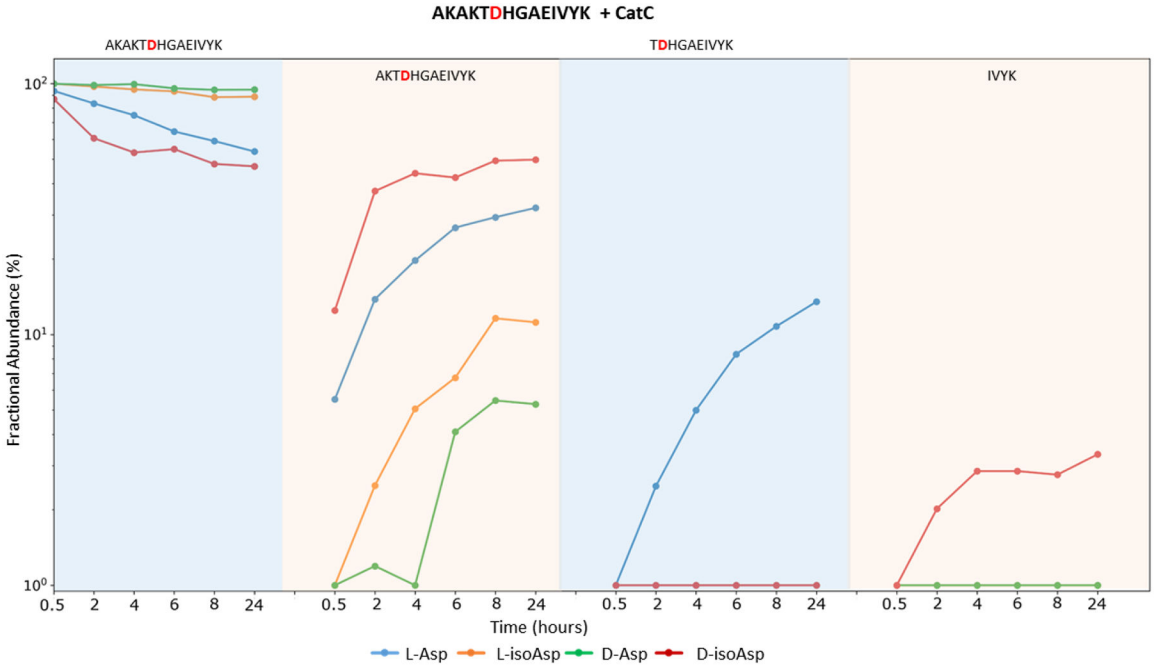


Figure 3.

Data is plotted for digestion of AKAKTDHAGEIVYK by di-amino peptidase cathepsin C at pH 6 using the same format outlined for Fig. 1. The data for the native form of the tau peptide shows enhanced proteolysis, as the enzyme cleaves past canonical-Asp yielding TDHGAEIVYK fragment, the fractional abundance of which increases with time. The D-isoAsp sample showed increased precursor degradation and an endopeptidase cut yielding IVYK fragment. L-isoAsp and D-Asp variants showed a markedly increased intact precursor and no internal cleavages. No digestion is observed near the isomerized Asp, in each of the three isomeric forms denoting the isomerized residues are resistant to proteolysis.

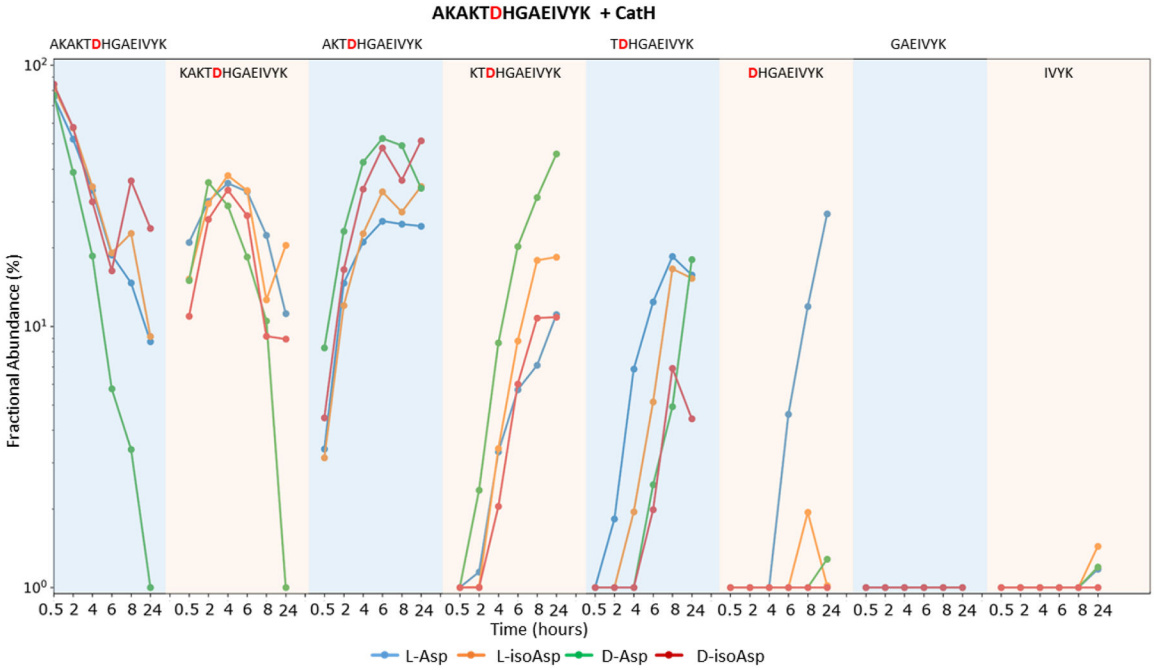


Figure 4.

Data is plotted for digestion of AKAKTDHAGEIVYK by cathepsin H (an endo/monoamino peptidase) at pH 5 using the same format outlined for Fig. 1. Each of the four isomeric forms show comparable fractional abundances for the exopeptidic cleavages away from the modified Asp. However, very low exopeptidase activity is observed beyond the isomerized residue, indicating that the placement of isomerized Asp residues impairs the CatH digestion.

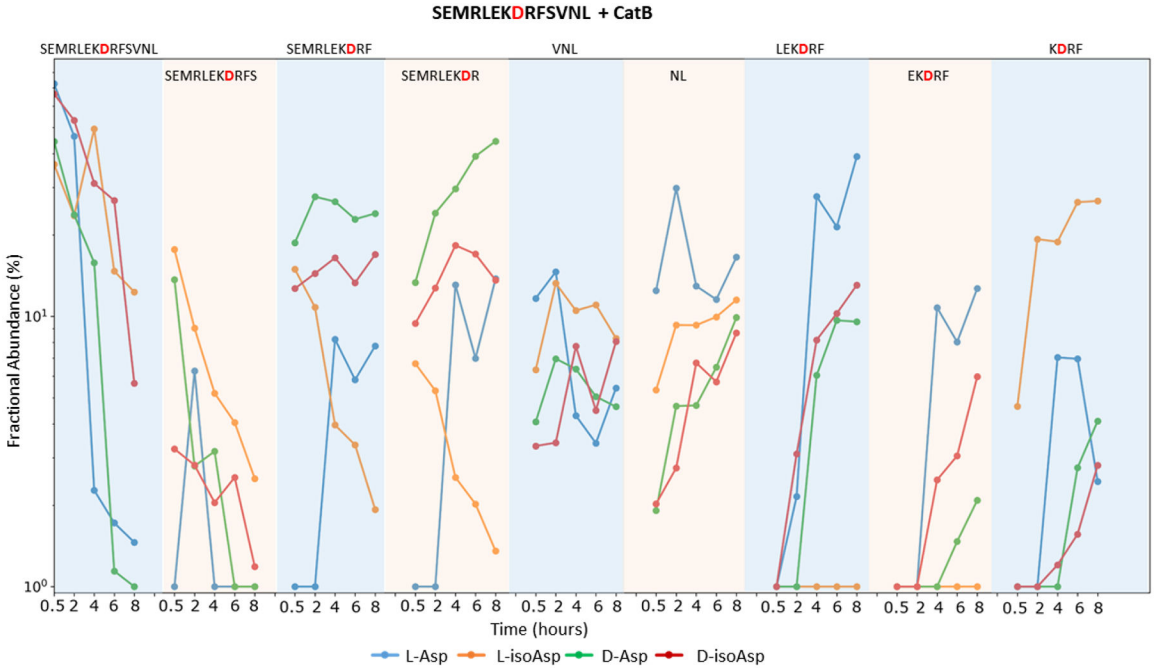


Figure 5.

Data is plotted for digestion of SEMRLEKDRFSVNL by di-carboxy peptidase CatB using the same format outlined for Fig. 1. The canonical and the L-Asp forms show higher levels of short proteolytic fragments (NL, VNL, EKDRF, KDRF), while D- and D-isoAsp exhibit higher levels of peptide fragments having longer chain-lengths (SEMRLEKDRF and SEMRLEKDR), indicative of the lasting resistance throughout the course of the digestion.

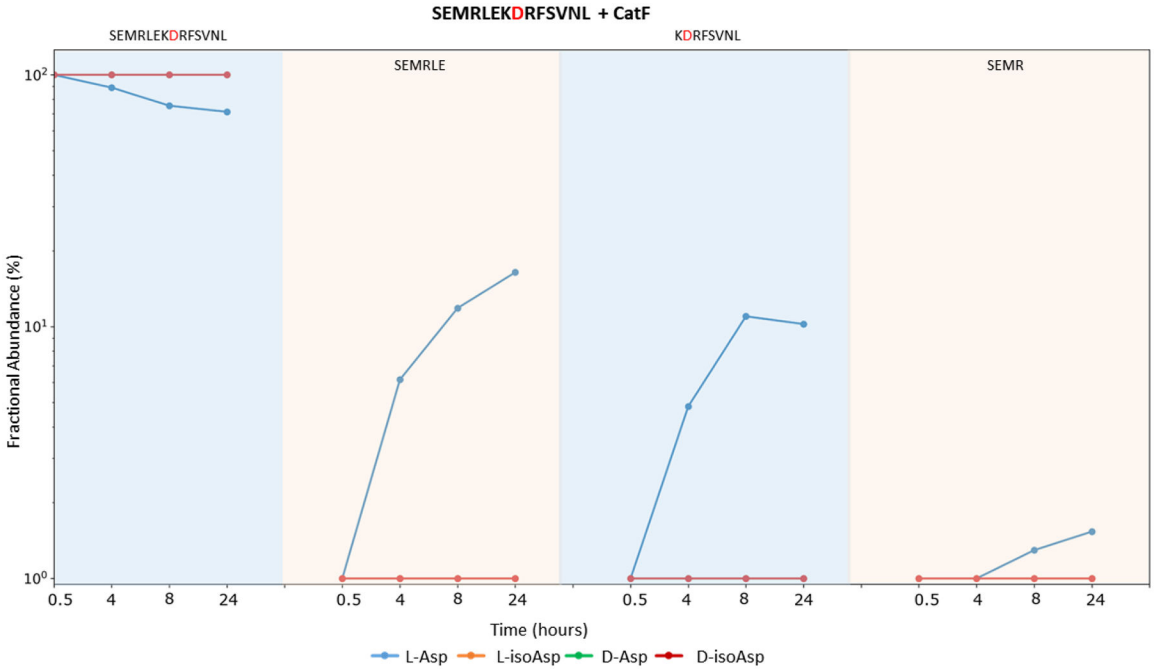
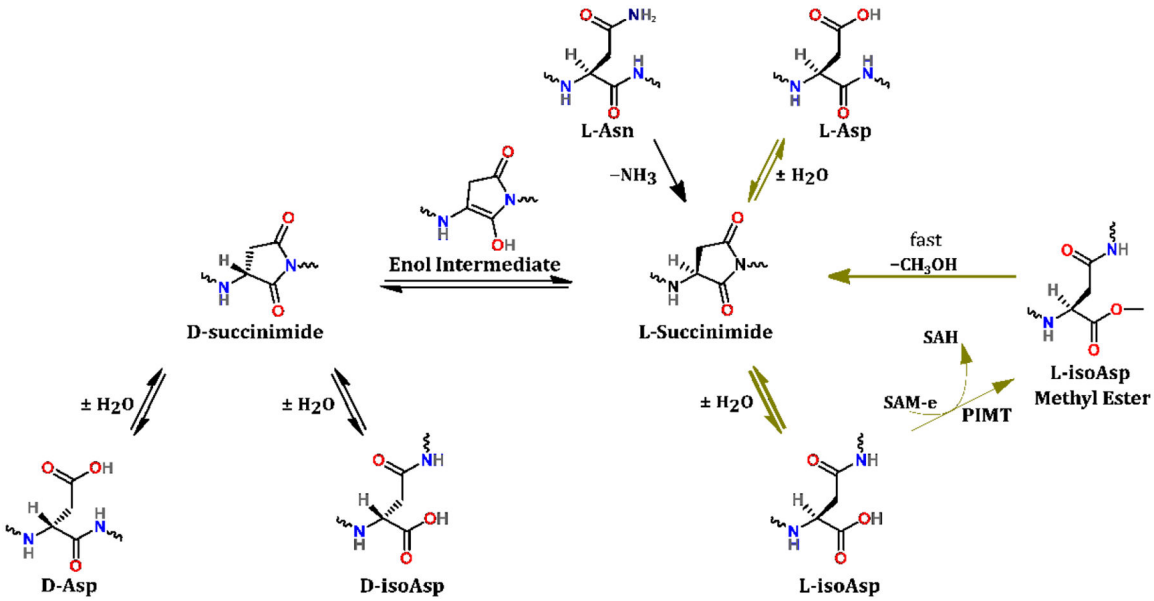


Figure 6.

Data is plotted for digestion of SEMRLEKDRFSVNL by cathepsin F at pH 5 using the same format outlined for Fig. 1. The all-L peptide data shows several internal cleavages, with their fractional abundance increasing with time, demonstrating the canonical form of the peptide is labile to CatF digestion. Contrastingly a completely intact precursor is observed for the non-canonical variants, as CatF fails to make any cleavages in the peptides bearing isomerized forms of aspartic acid. Note: all non-canonical results are identical and overlap.



Scheme 1.

Pathways for Isomerization of Aspartic Acid and Deamidation of Asparagine, and the protein-isoaspartyl methyl transferase (PIMT) catalyzed repair.