Cysteine cathepsins are essential in lysosomal degradation of α-synuclein

Ryan P. McGlinchey and Jennifer C. Lee1

Laboratory of Molecular Biophysics, Biochemistry and Biophysics Center, National Heart, Lung, and Blood Institute, Bethesda, MD 20892

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A cellular feature of Parkinson’s disease is cytosolic accumulation and amyloid formation of α-synuclein (α-syn), implicating a misregulation or impairment of protein degradation pathways involving the proteasome and lysosome. Within lysosomes, cathepsin D (CtsD), an aspartyl protease, is suggested to be the main protease for α-syn clearance; however, the protease alone only generates amyloidogenic C terminal-truncated species (e.g., 1–94, 5–94), implying that other proteases and/or environmental factors are needed to facilitate degradation and to avoid α-syn aggregation in vivo. Using liquid chromatography–mass spectrometry, to our knowledge, we report the first peptide cleavage map of the lysosomal degradation process of α-syn. Studies of purified mouse brain and liver lysosomal extracts and individual human cathepsins demonstrate a direct involvement of cysteine cathepsin B (CtsB) and L (CtsL). Both CtsB and CtsL cleave α-syn within its amyloid region and circumvent fibril formation. For CtsD, only in the presence of anionic phospholipids can this protease cleave throughout the α-syn sequence, suggesting that phospholipids are crucial for its activity. Taken together, an interplay exists between α-syn conformation and cathepsin activity with CtsL as the most efficient under the conditions examined. Notably, we discovered that CtsL efficiently degrades α-syn amyloid fibrils, which by definition are resistant to broad spectrum proteases. This work implicates CtsB and CtsL as essential in α-syn lysosomal degradation, establishing groundwork to explore mechanisms to enhance their cellular activity and levels as a potential strategy for clearance of α-syn.

Significance

Identifying factors that regulate the degradation of α-synuclein, the protein at the center of Parkinson’s disease etiology, is vital in designing therapeutic strategies. This study provides new mechanistic insights into α-synuclein clearance in the lysosome, a cellular site for proteolysis by using purified mouse lysosomes and purified lysosomal proteases. Cathepsins B and L are identified to be vital through peptide mapping by mass spectrometry. Importantly, cathepsin L degrades α-synuclein amyloid fibrils, materials associated with neurodegeneration, and, thus, changes in its activity or levels could provide a promising avenue for intervention. Additional results on the stimulatory effect of anionic phospholipids on cathepsin activity suggest that changes in lipid metabolism may also contribute to lysosomal dysfunction leading to pathogenesis.

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1To whom correspondence should be addressed. Email: lee4jl@mail.nih.gov.

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α-syn. Lysosomal and individual cathepsin activities on soluble, membrane-bound, and fibrillar α-syn as well as their impact on α-syn amyloid formation have been evaluated.

**Results**

**Mimicking α-syn Degradation by Lysosomes.** Purified lysosomes were isolated from mouse brain and liver and monitored for α-syn degradation (Fig. 1 and SI Appendix, Fig. S1). Reactions contained recombinant monomeric α-syn (α-synm, 3.8–60 μM) and lysosomal extract (18 μg total protein) in pH 5 buffer and were incubated at 37 °C (0–60 h). The addition of liver lysosomal extract completely inhibited α-synm (60 μM) aggregation as determined by no changes in turbidity (Fig. 1A) and emission intensity of an amyloid sensitive dye, thioflavin T (ThT) (Fig. 1B). Analysis by SDS/PAGE at 60 h revealed loss of full-length α-synm with generation of many smaller peptides (<2 kDa; Fig. 1B, Insert). In buffer alone, only full length protein is present and no degradation occurred during incubation (Fig. 1B, Insert). Transmission electron microscopy (TEM) confirmed that α-syn fibrils (α-synf) are not formed in the presence of lysosomal extract compared with the protein alone control (Fig. 1C).

To identify peptide fragments generated, LC-MS was performed at various incubation times (SI Appendix, Fig. S2 and Table S1). Cleavage sites (denoted by X/X, where / indicates the cut common to both sources) occurred mostly in the N-terminal amyloid region of α-syn at M5/K6, G14/V15, K34/E35, G41/S42, H50/G51, K60/E61, Q62/V63, G73/V74, T75/A76, A90/A91, and F94/V95 (Fig. 1D), which is consistent with the observed inhibition of α-syn aggregation.

**Cathepsin D Incompletely Degrades α-syn.** Unexpectedly, none of the major cleavage sites corresponded to previously reported CtsD activity (24, 30); therefore, we revisited α-syn degradation by CtsD. Upon incubation of α-synm with human CtsD at pH 5, two main species with molecular weights of ~12 and ~8 kDa were detected by SDS/PAGE and Coomassie staining (SI Appendix, Fig. S3A). LC-MS analysis of α-synm (2–60 μM) incubated with CtsD (0.1 μM) revealed peptide masses corresponding to fragments 1–94, 5–94, and 95–140, indicating the primary cleavage sites were F4/M5 and F94/V95 (SI Appendix, Fig. S3 B–D and Table S2). There are also minor cuts at M116/P117, A124/Y125, and G132/Y133. No further cleavage occurred within the amyloidogenic N-terminal fragment 5–94. Although the cleavage sites at F4/V5 and F94/V95 were not previously identified for CtsD (30), these cuts were found in low levels from lysosomal degradation of α-synm (Fig. 1D). Because in vivo experiments (21–24) rely heavily on immunoblotting with α-syn antibodies that have C-terminal epitopes such as the widely used Syn-1 (31), the fate of the N-terminal α-syn peptides (e.g., 5–94) as a result of CtsD activity would not have been previously observed (SI Appendix, Fig. S4). Hence, interpretations based on Western blot analysis that CtsD fully degrades α-syn in vivo are incorrect.

Aggregation of α-synm (3.8–60 μM) still occurs in the presence of CtsD, and the presence of fibrils were confirmed by TEM (SI Appendix, Fig. S5). Interestingly, N- and C-terminal truncations by CtsD did not accelerate aggregation as would be anticipated from previously reported data at neutral pH (10, 25, 26). Instead, the lag phases were unchanged with the exception of α-syn concentrations <15 μM, where the lag times actually lengthened. This may be explained by the presence of in situ-generated C-terminal peptide, which could modulate aggregation propensity, whereas previous studies used recombinant α-synΔC constructs. Nevertheless, CtsD alone cannot degrade α-synm and prevent fibril formation. In accordance, limited proteinase K (PK) digestion of fibrils at pH 5 showed the smallest PK-resistant core contains residues 19–89 (SI Appendix, Table S3), a region that CtsD does not cleave within. Clearly, these results strongly suggest the involvement of other proteases in lysosomal clearance of α-synm.

**Cysteine Cathepsins Are Responsible for α-syn Degradation.** To identify the class of protease(s) responsible for α-synm degradation, lysosomal extracts were treated with protease inhibitors. Of the four known protease types (serine, cysteine, aspartyl, and metalloproteases), we elected to focus on cysteine and aspartyl proteases because they represent the majority of lysosomal activity (19). α-synm in the presence of liver lysosomal extrs was treated with leupeptin (LeuP) and pepstatin A (PePA), cysteine and aspartyl protease inhibitors, respectively. In the presence of LeuP, the inhibition of α-synm aggregation by lysosomal extract was reverted, exhibiting both apparent lag and elongation phases (Fig. 2A; SI Appendix, Table S4). Corresponding LC-MS analysis (SI Appendix, Fig. S6 and Table S4) showed that the majority of cleavage sites were eliminated by the addition of LeuP to both liver and brain lysosomal extracts, supporting the observation that α-synm can now aggregate. The only activity from these samples resulted in cuts at F4/M5, F94/V95, N103/E104, and N122/E123 for brain and K6/C67, L8/S9, N103/E104, and E139/A140 for liver lysosomal extracts. F4/M5 and F94/V95 sites are consistent with CtsD activity, whereas cuts at N103/E104 and N122/E123 could be attributable to asparagine endopeptidase (AEP) activity. The presence of these cuts is revealed because AEP hydrolysis of substrates C-terminal of Asn residues is insensitive to LeuP (32). The origin of K6/C67, L8/S9, and E139/A140 cleavage sites could not be identified.

In contrast, samples with PePA behaved as the control (no inhibitor added) with no detectable changes even after 60 h (Fig. 2B). In the presence of PePA, most of the lysosomal activity was retained with the exception of cleavages at F4/M5 and F94/V95 (SI Appendix, Fig. S6 and Table S5). These data establish that
Lysosomal Cysteine Cathepsin Activity Involves CtsB and CtsL. Because cysteine cathepsins are involved in lysosomal degradation of α-syn, α-syn was incubated with liver lysosomal extract. In the presence of LeuP, a cysteine protease inhibitor (red), or PePA, an aspartyl protease inhibitor (green), respectively. (Inset) Western blot analysis of liver lysosomal extract probed with antibodies against endogenous CtsB (Upper) and CtsL (Lower). (B) Aggregation kinetics of α-syn (10, 20, and 40 μM) incubated with liver lysosomal extract with or without PC/BMP vesicles at pH 5 was monitored by ThT fluorescence (λex = 480 nm). (C) SDS-PAGE (4–12%) visualized by Coomassie staining showing degradation of α-syn (5, 10, and 20 μM) by 0.15 μM CtsB (green), CtsL (black), and CtsD (purple) after 2 min at pH 5 and 37°C. α-syn incubated alone is also shown. (D) Location of the corresponding cleavage sites generated by reactions shown in C as identified by LC-MS analysis (SI Appendix, Tables S7 and S8) are shown as triangles. Primary cleavage sites (denoted by X/X, where / indicates the cut) are highlighted.

Effect of Lipids on the Lysosomal Activity. Because membranes modulate α-syn conformation and its propensity to form amyloid (36), the effect of phospholipid vesicles on α-syn degradation by lysosomal extracts was investigated. Two lipids abundant in intraluminal vesicles in lysosomes (37, 38) were used, zwitterionic phosphatidylcholine (PC) and anionic bis(monoacylglycero)phosphate (BMP). Anionic lipids are of particular interest because α-syn prefers to associate with negatively charged membranes in forming α-helical structures (39). Secondary structural changes of α-syn were probed by circular dichroism spectroscopy. In solution, α-syn is highly disordered with a negative maximum at 198 nm (Fig. 3A, red). As expected, no apparent changes are detected upon the addition of 100% PC vesicles (Fig. 3A, gray lines), whereas spectroscopic signature of double-negative maxima (208 and 222 nm) affirms the formation of α-helical structure in the presence of BMP-containing (30 mol %) PC (PC/BMP; Fig. 3A, blue) vesicles (average diameter, ~100 nm).

The addition of PC/BMP vesicles to α-syn (20 μM and lipid-to-protein (L/P) molar ratio of 60) and liver lysosomal extracts yielded slightly less full-length and <12-kDa peptides (Fig. 3B and SI Appendix, Fig. S1A) compared with that in solution alone, suggesting a modest enhancement of degradation. This stimulatory effect is more evident in the data obtained from brain lysosomal extracts (SI Appendix, Fig. S1B). In contrast, the addition of PC vesicles to liver lysosomal extracts and α-syn inhibited activity slightly because <12-kDa peptides are more abundant than that of buffer alone (SI Appendix, Fig. S1A). These results show the impact of phospholipids on α-syn conformation and its degradation process. Activity from all three cathepsins was observed by LC-MS analysis (SI Appendix, Table S9).

Effect of Lipids on Individual Cathepsin Activity. To delineate whether aspartyl and/or cysteine cathepsins are modulated by the presence of PC/BMP vesicles, inhibition experiments were conducted (Fig. 3B). When LeuP is added to inhibit cysteine cathepsins, the degradation of the full length protein is suppressed and most of the lower-molecular-weight bands disappear with the exception of an 8-kDa fragment, which we attribute to CtsD activity. Consistently, lysosomal degradation of α-syn is unchanged upon PePA inhibition of aspartyl protease activity, namely CtsD. These results confirm that cysteine cathepsins are essential in lysosomal degradation of α-syn and indicate that cathepsin activity, especially CtsD, may be enhanced by anionic lipids.

To test the assertion that CtsD is stimulated by lipids, α-syn (5, 10, and 20 μM) was incubated with PC/BMP and PC vesicles at an L/P of 60 in the presence of purified CtsB, CtsL, and CtsD (0.15 μM) (Fig. 3C and SI Appendix, Figs. S12–S15). Changes in α-syn degradation were noticeable in the presence of PC/BMP vesicles (Fig. 3C). Consistent with the solution data, CtsL remains only observed at the highest α-syn concentration of 20 μM. Consistent results were also observed for an overnight reaction for 60 μM α-syn (SI Appendix, Fig. S8). LC-MS data taken at different times for the reactions of CtsB and CtsL with α-syn (SI Appendix, Figs. S9 and Tables S6–S8) were used to identify cleavage sites (Fig. 2D). Initial cuts were G14/V15 and A90/A91 for CtsB, M5/K6 and N103/E104 for CtsL, and F4/M5 and F94/V95 for CtsD. Remarkably, all cuts from lysosomal degradation can be assigned to a combination of CtsB, CtsL, and CtsD activity with the exception of K12/E13 and N122/E123 (previously suggested as AEP activity).

Distinct cleavage sites for CtsB (e.g., G14/V15 and A90/A91) and for CtsL (e.g., M5/K6 and N103/E104) affirmed both their presence in the lysosomal extracts. By comparing multiple LC-MS experiments from both lysosomal sources, brain lysosomal extracts harbor relatively more CtsB activity as noted by the significant presence of peptides 1–90 and 91–140. CtsB and CtsL activities were comparable in liver lysosomal extracts (SI Appendix, Fig. S10).

Fig. 2. Identification of cysteine cathepsins in lysosomal degradation of α-syn. (A) Aggregation kinetics of α-syn (53 μM) incubated with liver lysosomal extract (38 μg total protein) (black) in the presence of LeuP, a cysteine protease inhibitor (red), or PePA, an aspartyl protease inhibitor (green), respectively. (Inset) Western blot analysis of liver lysosomal extract probed with antibodies against endogenous CtsB (Upper) and CtsL (Lower). (B) Aggregation kinetics of α-syn (10, 20, and 40 μM) incubated with liver lysosomal extract with or without PC/BMP vesicles at pH 5 was monitored by ThT fluorescence (λex = 480 nm). (C) SDS-PAGE (4–12%) visualized by Coomassie staining showing degradation of α-syn (5, 10, and 20 μM) by 0.15 μM CtsB (green), CtsL (black), and CtsD (purple) after 2 min at pH 5 and 37°C. α-syn incubated alone is also shown. (D) Location of the corresponding cleavage sites generated by reactions shown in C as identified by LC-MS analysis (SI Appendix, Tables S7 and S8) are shown as triangles. Primary cleavage sites (denoted by X/X, where / indicates the cut) are highlighted.

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the most efficient protease in the presence of PC/BMP vesicles; however, CtsD proteolysis of α-syn$_{\text{m}}$ is dramatically stimulated by PC/BMP and is now comparable to CtsB. We note that there is also a small enhancement in CtsB and CtsL degradation because the abundance of α-syn$_{\text{f}}$ remains in the presence of CtsB, it is a direct modulation of the active site by lipids or perhaps enhancement arises because of an increase in the effective concentration on the membrane surface. More work is clearly needed to discern what factors are involved in the stimulation mechanism of CtsD by anionic lipids.

**Cathepsin L Degradates α-syn Fibrils.** Because both CtsB and CtsL efficiently degraded monomeric α-syn, we investigated whether they would also have capabilities in digesting preformed fibrils. For comparison, CtsD was also tested. Remarkably, CtsL degraded α-syn$_{\text{f}}$ (20 μM), whereas both CtsB and CtsD had moderate effects (Fig. 4). Significant losses were observed in both turbidity and ThT fluorescence (Fig. 4A and B) after 5 h in the presence of CtsL. The amount of proteins that persist is miniscule (Fig. 4C and SI Appendix, Fig. S1 and S2), and no intact fibrils were found by TEM (Fig. 4D). For comparison, TEM image of α-syn$_{\text{f}}$ alone is also shown in Fig. 4D. Although α-syn$_{\text{f}}$ remains in the presence of CtsB, it is noteworthy that TEM images indicate thinner fibrils, suggesting that the enzyme is trimming solvent accessible regions. Supporting this assertion, remaining peptide fragments identified by LC-MS (e.g., 15–114 and 20–114; SI Appendix, Table S13) still retained the PK-resistant core. Similarly, some degradation by CtsD also did occur (Fig. 4B and SI Appendix, Fig. S14); fragments were identical to those observed with α-syn$_{\text{m}}$ (SI Appendix, Table S2). In contrast to α-syn$_{\text{m}}$, the inclusion of anionic lipids did not have a dramatic effect on CtsD activity on α-syn$_{\text{f}}$ (SI Appendix, Fig. S16).

Our data suggest that only CtsL is capable of disassembling α-syn$_{\text{f}}$. To test whether there is synergy between the enzymes, we performed experiments using liver lysosomal extracts. Although there was an initial drop in turbidity, no differences were observed with further incubation (SI Appendix, Fig. S17). ThT fluorescence and SDS/PAGE showed similar results relative to the α-syn$_{\text{f}}$ were common in the presence of both PC/BMP (SI Appendix, Table S10) and PC (SI Appendix, Table S11), the abundance of these generated peptide fragments were significantly different (SI Appendix, Fig. S15). Interestingly, CtsD prefers to cleave within the C terminus in the presence of PC vesicles and unique sites were observed (Fig. 3D).

We infer from these data that α-syn conformation may be less important than BMP stimulation of CtsD activity. It is plausible that there is a direct modulation of the active site by lipids or perhaps enhancement arises because of an increase in the effective concentration on the membrane surface. More work is clearly needed to discern what factors are involved in the stimulation mechanism of CtsD by anionic lipids.

**Fig. 3.** Effect of phospholipids on cathepsin degradation of α-syn. (A) Far-UV CD spectra of α-syn$_{\text{m}}$ (5 μM) in the presence of PC (gray) and PC/BMP (yellow to green-blue) vesicles at varying lipid concentrations (lipid-to-protein molar ratio [L/P] = 1, 5, 10, 20, and 60) at pH 5 and 37 °C. (B) SDS/PAGE (4–12%) of preformed α-syn$_{\text{f}}$ (20 μM) incubated with PC/BMP and PC alone is also shown. (C) Schematic representation of the primary amino acid sequence of α-syn$_{\text{m}}$ with identifiable cleavage sites generated from CtsD activity in the presence of PC (magenta and black) or PC/BMP (black) vesicles (SI Appendix, Tables S10 and S11); α-syn membrane binding region is depicted as spanning residues 1-100, whereas C-terminal residues 101-140 are denoted as the acidic region.

**Fig. 4.** Effect of cathepsins on α-syn fibril stability. (A) Turbidity measurements (λ$_{\text{obs}}$ = 440 nm) of preformed α-syn fibrils (α-syn$_{\text{f}}$, 20 μM) incubated with 0.15 μM CtsB (purple), CtsL (green), and CtsD (black) as a function of time at pH 5 and 37 °C. (B-D) Representative ThT fluorescence (error bars represent SD; n = 3) (B), SDS/PAGE (4–12%) (C), and TEM images (D) taken after 20 h of incubation. Scale bars are as indicated. Results for α-syn$_{\text{m}}$ alone are also shown in red.
control, indicating little change in the amounts of α-syn (SI Appendix, Fig. S17). The change in turbidity could be attributable to some fibril proteolysis but insufficient to change ThT intensity. In accordance, LC-MS analysis shows only minor amounts of α-syn peptides were generated (SI Appendix, Table S15). The addition of lipids did not have a significant effect (SI Appendix, Fig. S17). A concentration dependence of purified CtsL on α-syn fibril degradation (SI Appendix, Fig. S18) indicate that results obtained with lysosomal extracts are comparable to those obtained for nanomolar CtsL (<9.4 nM), which are not sufficient to fully digest the fibrils under these experimental conditions.

Discussion

Accumulation of aggregated α-syn is a feature in PD pathogenesis, and therefore enhancement in lysosomal degradation could prove beneficial. Our work points to a direct role for cysteine cathepsins in lysosomal clearance of α-syn. Cysteine cathepsins CtsB and CtsL alone are capable of proteolyzing the central and amyloidogenic region of α-syn. In contrast, the current cathepsin thought to be responsible for lysosomal degradation of α-syn, CtsD, requires the presence of anionic lipids to degrade that same region. Interestingly, CtsL effectively degrades α-syn amyloid fibrils, materials that are closely associated with neurodegeneration. Collectively, this work offers a new perspective on how lysosomes deal with α-syn, where the protein conformational state dictates the role of individual cathepsins (Fig. 5). Specifically, soluble and membrane-bound α-syn is readily digested by both CtsB and CtsL, whereas CtsD is only effective in processing the membrane-bound form. Finally, CtsL would be capable of proteolyzing aggregated α-syn. Whereas CtsD activity is stimulated by anionic lipids in vitro, CtsL is still the most efficient, with CtsD comparable to CtsB activity under these conditions. Even in the presence of anionic lipids, CtsD activity in lysosomal extracts is insignificant compared with that of cysteine cathepsins (Fig. 3B). Thus, we conclude cysteine cathepsins are essential in the lysosomal degradation of α-syn.

Cysteine cathepsins have been associated with other neurodegenerative ailments (34), including amyloid disorders such as Alzheimer’s (33) and prion diseases (35). Evidence directly linking these proteases to α-syn has not been demonstrated until now. Interestingly, one study showed an abnormal distribution of CtsL in postmortem human brain tissues of PD patients (41), and in another, treatment with a potent cysteine cathepsin inhibitor, cystatin C, induced insoluble α-syn accumulation in primary cultured neurons from mice (42). Because lysosomal malfunction is associated with PD, we propose that CtsB and CtsL could act as disease modifiers.

The finding that cysteine cathepsins are essential in lysosomal degradation of α-syn is in contrast with previous literature reporting CtsD as the main protease involved (21–24). We propose different interpretations of prior literature that might reconcile these differences. First, the reliance on C-terminal α-syn antibodies to monitor degradation of α-syn gives no information regarding the fate of the N-terminal amyloidogenic region because this epitope is lost during CtsD activity. Hence, the conclusion that CtsD is the protease responsible for complete degradation of α-syn needs readjusting. Second, a more indirect role for CtsD is plausible such as CtsD activation of CtsB (43, 44), which then is responsible for α-syn proteolysis. Another indirect mechanism involves CtsD degradation of cystatins (45), inhibitors of cysteine cathepsins, which would enhance CtsB and CtsL activity upon CtsD up-regulation.

This work also reveals an important role for phospholipids in α-syn degradation. As lipid metabolism changes with age and/or with disease (46–48), it is thus conceivable that subtle alterations in phospholipid membrane compositions could impact not only membrane binding of α-syn but also its proteolysis and could thereby enhance α-syn accumulation and aggregation in the lysosome. In a broader context, membranes may also play a role in the turnover of other aggregation-prone proteins. Proteo-lipid interactions could have both positive and negative effects in modulating proteolysis efficiency, depending on what peptide regions are exposed to proteases and whether the resulting fragments have reduced or increased aggregation propensity. Finally, membranes themselves could either promote or inhibit protein aggregation, which would further impact proteostasis.

Further investigation is clearly needed to determine whether and to what extent CtsB and CtsL and/or the interplay among the cathepsins is involved in PD. Of particular interest would be the quantification of the physiological concentrations of individual cathepsins as well as their activities through careful proteomics characterization of brain samples of PD patients and healthy individuals. Nevertheless, with the identification of CtsB and CtsL, increasing their activities and/or levels should have positive results toward the clearance of α-syn. This would offer a targeted therapeutic strategy which could have advantages over a general modulator of autophagy. Future in vivo studies modulating CtsB and CtsL will further demonstrate whether cysteine cathepsins would be able to ameliorate α-syn pathology and should provide insights into relevant therapeutic strategies.

Materials and Methods

Details regarding proteins and reagents, experimental methods for lysosomal extract and lipid vesicle preparations, cathepsin activity assays, TEM, gel electrophoresis and immunoblotting, and CD spectroscopy are provided in the SI Appendix. All animal studies were approved by the National Heart, Lung, and Blood Institute (NHLBI) Animal Care and Use Committee (protocols H#-0050 and H-0018).

Fibril Formation. α-syn was exchanged into pH 5 buffer (20 mM sodium acetate (NaOAc); 50 mM NaCl) using a PD-10 column (GE Healthcare) and filtered through Amicon Ultra 0.5 L Ultrasole 30k membrane centrifugal filters (Millipore) to remove any preformed aggregates before aggregation. Aggregation was performed in sealed clear, polystyrene, 384-well flat-bottom microplates (781185; Greiner Bio-One) containing 40 μL of solution (α-syn, 50–60 μM) with continuous orbital shaking (1.0 mm, ~87.6 rpm) at 37 °C using a microplate reader (Tecan Infinite M200 Pro). Turbidity (440 nm) and ThT (10 μM) fluorescence (excitation and emission wavelengths at 415 and 480 nm, respectively) was recorded as a function of time. For experiments with lysosomal extracts and purified cathepsins, buffer also contains 5 mM EDTA and 5 mM DTT.
LC-MS. Samples (18 μL) were separated using a Vydac 218TP C18 reverse-phase column (2.1×50 mm; 5 μm) and an Agilent 1100 series HPLC (Agilent Technologies) coupled to an Agilent G1946D mass selective detector (MSD) equipped with an electrospray ionization interface (Agilent Technologies). Mass spectra were obtained using an ion mode. The HPLC system and MSD were controlled and data were analyzed using LC/MSD ChemStation software (Rev. A.10.02. Agilent Technologies).

Degradation Rate of α-syn. In microcentrifuge tubes (Safe-Lock tubes; 1.5 mL Eppendorf), α-syn (2–60 μM) was incubated with cathepsin B (0.15–0.15 μM) in reaction buffer (20 mM NaOAc, 50 mM NaCl, 5 mM DTT, 5 mM EDTA; pH 5) in a total volume of 40 μL and aliquoted at 300 rpm at 37 °C in a Mini-Micro 980140 shaker (VWR). Reactions were terminated with either PePA (1 μL 73 μM) or LeuP (1 μL 100 μM) at various time points. For lysosomal extracts, 18 μg of total protein was used. The same conditions were performed for α-syn (20 and 60 μM) reactions. To prepare fibrils, α-syn (144 μM) was agitated at 300 rpm in microcentrifuge tubes for 3 d at pH 5 and 37 °C in a Mini-Micro 980140 shaker (VWR). Aggregated samples were centrifuged at 16,100 × g for 10 min, and the supernatant was discarded. Fibril pellets were resuspended in reaction buffer at desired concentration. Fibril disassembly was performed in sealed clear, polystyrene, 384-well flat-bottom microplates (781185, Greiner Bio-One) containing 40 μL of solution (α-syn), 20 and 60 μM incubated with continuous orbital shaking (1.0 mm; ~8.76 rpm) at 37 °C using a microwell reader (Tecan Infinite M200 Pro). Turbidity (440 nm) was recorded as a function of time.

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