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Unaided trifluoroacetic acid pretreatment solubilizes polyglutamine peptides and retains their biophysical properties of aggregation

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ABSTRACT

Understanding the biophysical mechanism of polyglutamine (polyGln) aggregation is important to unravel the role of aggregates in the pathology of polyGln repeat disorders. To achieve this, synthetic polyGln peptides are widely used. Their disaggregation and solubilization is essential because it plays a crucial role in reproducing biophysical experimental data under *in vitro* conditions. Pretreatment with trifluoroacetic acid (TFA) and hexafluoroisopropanol (HFIP) at a 1:1 ratio is currently the method of choice to achieve solubility of polyGln peptides. Here we report that the disaggregation and solubilization of polyGln peptides can be achieved by TFA alone. We tested TFA due to the close similarity of it with HFIP in the nature of H-bond breakage and formation, higher cost, and the problems faced by us in the availability of HFIP. Our results demonstrate that the TFA disaggregated polyGln sequences give similar solubilization yield, aggregation kinetics, thioflavin T (ThT) binding, and structural features in comparison with the TFA/HFIP method. Furthermore, we show by limited validation studies that the proposed TFA method can replace the existing TFA/HFIP disaggregation method of polyGln sequences.

Protein aggregation is associated with at least 20 diseases of brain, systemic, and localized origin. Some of the proteins/polypeptides involved are beta-amyloid peptide, α-synuclein, prions, polyglutamines, insulin amyloid polypeptide, and transthyretin [1]. Nine different polyglutamine-containing proteins are involved in Kennedy disease, different forms of ataxia (types 1, 2, 3, 6, 7, and 17), and Huntington's disease [1,2]. The expansion of polyglutamine (polyGln) length in mutant huntingtin (mHtt) above the threshold limit of approximately 35 residues due to corresponding CAG expansion mutation in the huntingtin (IT15) gene is responsible for Huntington's disease [3].

In vitro approaches have been used extensively to examine protein aggregation [4–19]. A major difficulty faced in these studies is the preparation of monomeric solutions of aggregation-prone proteins and peptides. This step is required to ensure that aggregation is initiated from the same starting conditions. Inadequate solubilization can lead to misinterpretation of kinetics and thermodynamics of aggregating reactions.

To resolve the solubility problem, nonvolatile denaturing solvent such as dimethyl sulfoxide (DMSO) and volatile denaturing solvents such as trifluoroacetic acid (TFA), hexafluoroisopropanol (HFIP), and a combination of both (TFA/HFIP) have been tested for their ability to disaggregate and solubilize insoluble synthetic peptides [20]. The use of DMSO is imperfect due to its inability to solubilize the synthetic peptides completely. Besides, its continuous presence in the reaction mixture may lead to biased results. Hence, for more than a decade, the TFA and HFIP combination had been the preferred choice for disaggregating polyGln-containing peptides.

The biophysical understanding of polyGln aggregation was derived from synthetic polyGln peptides of different lengths [21]. These studies were possible because of the development of a robust procedure where synthetic polyGln peptides are pretreated for 3 h to overnight in a 1:1 ratio of TFA/HFIP at room temperature. After incubation, volatile solvents are evaporated, followed by a vacuum drying step. The obtained disaggregated peptide residue is solubilized in water–TFA (pH 3.0) [22,23]. This procedure improved the solubilization of otherwise insoluble polyGln peptides. It further

Abbreviations used: polyGln, polyglutamine; DMSO, dimethyl sulfoxide; TFA, trifluoroacetic acid; HFIP, hexafluoroisopropanol; ThT, thioflavin T; PBS, phosphate-buffered saline; RP-HPLC, reversed-phase high-performance liquid chromatography; TM–AFM, tapping mode–atomic force microscopy; TEM, transmission electron microscopy; FTIR, Fourier transform infrared; RSD, relative standard deviation.

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resolved the problem of non-reproducibility of experimental data reported for similar peptides [7,24,25].

We noticed that the cost of HFIP per milliliter is higher than that of TFA per milliliter. In addition, we observed that HFIP availability at times is low. Moreover, both TFA and HFIP may act in a similar fashion for making or breaking H-bonds during disaggregation of insoluble peptides (see Table 1 in Ref. [26]). Therefore, we tested anhydrous TFA alone and compared it with the TFA/HFIP method of polyGln disaggregation. Based on the experimental evidence, we demonstrate here that the recovery of peptides after disaggregation, overall kinetics, and structure of aggregates formed remain the same after disaggregation with TFA when compared with the TFA/HFIP method.

Materials and methods

Materials

All of the peptides (Table 1) were procured in crude from the Keck Biotechnology Center at Yale University. TFA, thioflavin T (ThT), and HFIP were purchased from Sigma–Aldrich, and sodium azide was purchased from Sd Fine Chemicals. Phosphate-buffered saline (PBS) was prepared as per the standard procedure described in Cold Spring Harbor protocols.

Peptide design

Table 1 shows the peptides chosen for this study. Peptides are free at the N and C termini. Q_{35} peptide was chosen as a representative of simple polyGln peptides. Because the lag time of polyGln aggregation is length dependent [12], PGQ_9Q, a 46-residue polyGln peptide representing a model peptide of β-sheet formation, was chosen [27]. The role of flanking sequences, NT_{17} and P_{10}, in polyGln aggregation was evaluated with the help of $NT_{17}Q_{35}P_{10}$ and $Q_{35}P_{10}$ peptides, respectively. NT_{17} is expected to enhance [13], and P_{10} delays [9,15], the rate of polyGln aggregation. In this study, $NT_{17}Q_{35}P_{10}$ was chosen based on its ability to aggregate as a full-length exon1 encoded huntingtin sequence [28]. We also chose PGQ_9I, a variant of PGQ_9Q, to understand the effect of isoleucine (I) substitution mutation on polyGln aggregation (R. Mishra and A. K. Thakur, unpublished data).

Methods

Peptide purification and storage

Approximately 1 mg (analytical balance, Sartorius BSA 224S-CW) of crude peptide was dissolved in 200 μl of formic acid. It was then adjusted to 20% formic acid with high-performance liquid chromatography (HPLC)-grade water before injecting into a Zorbax SB C_3 semi-preparative column (9.4 × 250 mm) attached to a Bio-Rad (Biologic Duoflow) fast protein liquid chromatography system for purification. The pure fraction of peptide was collected in one glass vial (Borosil, 15 ml) based on the retention time of the main peak that was earlier characterized by mass spectrometry. Subsequently, it was dried by lyophilization and stored at −80 °C for future use [22,29].

Table 1
PolyGln containing peptide sequences chosen for the disaggregation and solubilization study.

Peptide	Amino acid sequence	Sequence length
Q_{35}	K_2-Q_{35}-K_2	39
PGQ_9Q	K_2-Q_9-PG-Q_9-PG-Q_9-PG-Q_9-K_2	46
$NT_{17}Q_{35}P_{10}$	MATLEKLMKAFESLKSF-Q_{35}-P_{10}-K_2	64
$Q_{35}P_{10}$	K_2-Q_{35}-P_{10}-K_2	49
PGQ_9I	K_2-Q_9-PG-Q_4-I-Q_4-PG-Q_9-PG-Q_9-K_2	46

Disaggregating polyGln-containing peptide sequences

Q_{35}, $Q_{35}P_{10}$, PGQ_9Q, and PGQ_9I peptides were disaggregated under two different conditions: (i) overnight incubation in a 1:1 ratio of TFA/HFIP at 0.5 mg/ml peptide concentration [23,29] and (ii) overnight incubation in only TFA at 0.5 mg/ml concentration. In both conditions, the final concentration maintained for $NT_{17}Q_{35}P_{10}$ peptide was 0.1 mg/ml. This is because its disaggregation at a lower concentration improves recovery of this peptide [22,29]. After overnight incubation, the volatile solvents were evaporated in a chemical fume hood using a gentle stream of nitrogen gas, producing a thin peptide film on the glass walls. To further ensure the complete removal of any residual TFA or TFA/HFIP, they were subjected to drying under vacuum in a desiccator for approximately 1 h. The peptide film was then solubilized in water–TFA (pH 3.0) and subjected to gentle swirling to ensure complete solubility [23,29]. Visual inspection showed no cloudiness or insoluble aggregates, suggesting a clear and transparent solution in both conditions.

Comparison of yield after disaggregation under TFA and TFA/HFIP conditions

Peptide solubilized in water–TFA was ultracentrifuged at 305,611 *g* and 4 °C for 4 h to remove insoluble peptide or micro-aggregates. Two-thirds of the supernatant was carefully removed, and the peptide concentration of the supernatant was determined by injecting in an Agilent Eclipse Plus C_{18} column (4.6 × 100 mm) connected to a reversed phase (RP)–HPLC device. This was achieved by comparing the observed peak area with the standard curve generated for each peptide. The standard curve was generated from the stock concentration that was determined based on the molar extinction coefficient of peptides at 214 nm in an ultraviolet (UV) spectrophotometer [10,30]. The determined concentration was then converted to percentage yield by dividing the supernatant peptide concentration (mg/ml) by the concentration of the initial sample prior to ultracentrifugation. To determine the effect of time of disaggregation, PGQ_9I peptide was dissolved in TFA at 0.5 mg/ml for 1–12 h, dried, and resuspended in pH 3.0 water. Solubility was determined as described. To determine the effect of concentration during disaggregation, PGQ_9I was dissolved in TFA at various concentrations (0.3–2 mg/ml) for 12 h and solubility was determined as described.

Spontaneous aggregation kinetics analysis

The aggregation reaction setup and sedimentation assay were carried out as described previously [22]. All of the aggregation reactions were monitored at pH 7.2 in PBS at 37 °C. Sodium azide (0.05%) was added to the reaction mixture to avoid microbial contamination. The aggregation kinetics was monitored by taking supernatant from an aliquot of ongoing aggregation reaction after subjecting it to ultracentrifugation at 305,611 *g* and 4 °C for 30 min. The leftover monomer concentration at regular time intervals was determined by the RP–HPLC method [29,30].

ThT and light scattering assays were performed to corroborate the aggregation kinetics monitored by sedimentation assay [5]. In a 1-ml cuvette, an aliquot of 600 μl sample from ongoing reaction was taken at different time points. The intensity of light scattering was measured by setting both the excitation and emission wavelengths at 450 nm (both of the slit widths were adjusted to 2.5 nm at a voltage of 650). To this, 20 μl of 2.5 mM ThT was added and the spectra were recorded by resetting the excitation and emission wavelengths to 450 nm (slit width of 5 nm) and 489 nm (slit width of 10 nm), respectively, on a PerkinElmer model LS 51 spectrofluorimeter. The final spectra were obtained after averaging over three scans for each spectrum. The light scattering and ThT intensity was normalized against blank and then converted to